

# PROCEEDINGS OF SYMPOSIUM ON PROTEINS

*August 14-16, 1960*

HELD AT  
CENTRAL FOOD TECHNOLOGICAL RESEARCH INSTITUTE  
MYSORE

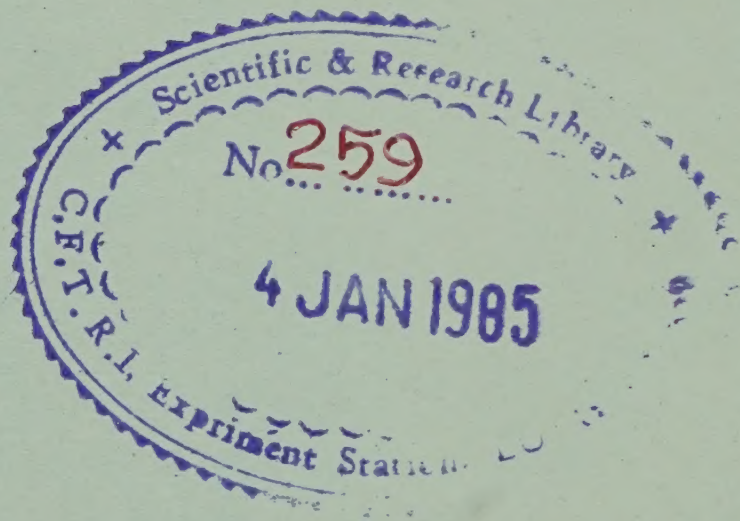


CHEMICAL RESEARCH COMMITTEE  
AND  
SOCIETY OF BIOLOGICAL CHEMISTS, INDIA



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## PREFACE

Proteins, in the form of enzymes, hormones, viruses, antibodies, are recognised to have specific functional significance in the chemical events of normal and pathological life processes. Much of the knowledge about them has been gained only in the last decade or two and has served practical ends in the fields of nutrition and medicine and in the industry. Since the symposium on 'Proteins' held in August 1954 under the auspices of the National Institute of Sciences of India, considerable progress has been made, including the application of results of research, necessitating another discussion of this vast subject.

The Chemical Research Committee of the C.S.I.R., therefore, decided at its meeting held in January 1960 to hold a symposium on Proteins under its auspices at the C.F.T.R.I., Mysore, and took a welcome decision to organize it jointly with the Society of Biological Chemists, India.

The invitation received a good response and over 80 papers were received from scientists engaged in research on different aspects of proteins.

The three-day symposium was inaugurated by Prof. M. S. Thacker, Secretary, Union Ministry of Scientific Research and Cultural Affairs and Director-General of Scientific and Industrial Research, on 14th August 1960 at a function presided over by Sri P. A. Narielwala, Director of Tata Industries.

The symposium was attended by a large number of delegates from different parts of the country and more than 50 papers from about 150 authors were presented and discussed. The papers were grouped under the following heads: (1) Chemistry and Methodology, (2) Biochemistry covering Biosynthesis, Metabolism and Inter-relationships, Immunological, Microbiological and Other Aspects, (3) Technology covering Protein Isolates, Protein-rich Foods, Protein Hydrolysates and Other Aspects, and (4) Nutrition covering Nutritional Evaluation and Amino Acid Composition, Human Nutrition and Therapy and Other Aspects.

An attempt has been made here to bring under one cover the proceedings of the symposium. The papers have been given in detail with a gist of the discussions. The symposium afforded ample evidence to show that Indian scientists are working on fundamental aspects of the problem as well as seeing through the application of this knowledge in meeting the more serious and urgent practical problems of combating the widespread protein malnutrition in the country. It is hoped that this collection will be of great interest and value to biochemists, nutritionists, dieteticians, technologists, industrialists, etc.

Our grateful thanks are due to the C.S.I.R. and particularly to Prof. M. S. Thacker for his encouragement, and to Dr B. C. Guha, Chairman of the Chemical Research Committee and the Society of Biological Chemists for their welcome decision to hold the



symposium at the Institute. We also thank those who contributed so much to the organization and success of the symposium, to the authors who presented the papers and to others who participated in the proceedings.

Our special thanks are due to Drs M. Srinivasan, D. S. Bhatia, R. Rajagopalan for assistance in editing some of the papers and to Messrs. A. Rahman, M. V. L. Rao, B. V. Subbarayappa, J. V. Shankar and K. L. Radhakrishnan for editing and seeing the Proceedings through the Press.

*A. Rahman*



**INAUGURAL SESSION**

*President*

SHRI P. A. NARIELWALA

*Inauguration*

PROF. M. S. THACKER







*Dr V. Subrahmanyam*

Shri Narielwala, Prof. Thacker, Dr Guha, Shri Vasudeva Rao, Colleagues from different parts of the country, Ladies and Gentlemen,

My colleagues on the organising committee have assigned to me a very safe and comfortable job. Early this year, when we, the members of the Chemical Research Committee, explored the possibilities of organising an All-India symposium, we were quite conscious that there would be no better subject than the present one. We realised, at the same time, that the subject is of tremendous importance and one which presents many fresh facets. In fact, right here, in the audience, there are some colleagues who will be leaving soon to attend the International Congress on Nutrition, and also the meeting of the Protein Advisory Group, where the very same themes are going to be discussed and, in fact, it is a matter for pride that the Indian workers are not only going to figure prominently, but the contributors from India will be asked to lead some of the discussions.

Years ago, when many of us studied Biochemistry, the word 'Protein' was no more than the name as to many others. They represented to the Chemist,  $N \times 6.25$  and not much more. In the past few decades there have been such tremendous developments, that now we know a good deal about these comparatively complicated and obscure chemical compounds which form a large part of the real active material in living tissues. If one may give an analogy, a discussion of proteins is like a literary discussion on Shakespeare. The same is true of that very fascinating groups of compounds, the nucleo proteins, on which possibly a few hundred schools are working in different parts of the world, doing excellent work and contributing many lines of thought. It is a very happy circumstance that Society of Biological Chemists is sharing in the programme and contributing to the success of it today. The Society is taking an active part in programmes of this type.

Since the inception of this Institute, by a curious dispensation, we have had more to do with proteins than probably any other form of food material. In the earlier days, we had to think of how to consume the minimum quantity of proteins, and later, our interests were confined to the richer sources, processing them and making them available to our people who needed them very badly. In fact, the biggest contribution of recent years has been the realisation that protein malnutrition is the major cause of a large variety of human ailments.

The Indian Institute of Science was probably one of the first schools to start research work on proteins, under the guidance of the late Dr Norris. A succession of workers followed in later years. One of the earlier attempts was to produce protein isolates for industrial use. It was, what we may consider, a very crude attempt, but it showed certain possibilities. Our own efforts were defective, but the subject got on very quickly into other parts of the world, and there was large-scale production. I am happy to see here Mr Srinivasan, one of the very first workers, and also Mr B. G. Krishnamurthy and Mr Srinivasaya, some of the earlier workers in India.

Extensive protein malnutrition and active developments in the field of production and utilization of isolates, has opened out a vast vista of possibilities. At one time, we



thought that animal proteins are superior to these isolates. It is now increasingly coming home to us that these are not only pure and concentrated but can also be processed, modified, enriched and combined so as to make something equal to/or superior to animal proteins. About two years ago, when this idea of using isolates for human therapy was mooted, there was no interest. Now International Organizations are not only very much alive to it but are extremely interested in the development of this line which has far-reaching possibilities.

We are happy that we have a distinguished leader in Mr Narielwala who is with us today to preside over the occasion. The Chief Minister was extremely keen to come, but he is so busy that he had to depute Shri Vasudeva Rao, the Divisional Commissioner and we are very fortunate in having him to read the message. We are also happy in having the Head of our family, Prof. M. S. Thacker, in our midst today, and it is a matter of great pride that a distinguished Scientist like himself has also become a leader of the Commonwealth Scientific Organization. Prof. B. C. Guha is, of course, the active brain behind. Dr Khanolkar is detained in Bombay, in connection with the University work and Dr Ramakrishnan will read his address. Shri Vasudeva Rao not only accepted our invitation at very short notice but has agreed very kindly to present the Chief Minister's message.

*Dr B. C. Guha*

Shri Narielwala, Prof. Thacker, Dr Subrahmanyam, Shri Vasudeva Rao, Ladies and Gentlemen,

I did not realise that I was put down in the programme of the inaugural meeting to deliver an address. As a matter of fact, all I expected was that I may be called upon to thank the CSIR for having approved the proposal of holding the symposium under the auspices of the Chemical Research Committee, and also to thank Dr V. Subrahmanyam, convener of the Organising Committee, and his very active and indefatigable collaborator, Dr A. Sreenivasan, for having made exhaustive preparations to make the symposium a success. Since I have been so ordered by the convener of the Organising Committee, I would like to say a few words, more or less similar to what Dr Subrahmanyam has already outlined.

I do not think the Chemical Research Committee could have chosen a more important subject for the symposium. As you know, perhaps, the origin of life itself was in proteins. Perhaps, in times past, nitrogen, hydrogen, oxygen, combined in some way forming amino acids. Proteins have been considered to be the physical basis of life itself. Even the smallest living organisms mainly consist of nuclear proteins, and they together carry life from generation to generation. And the protein exhibitions that you see are really due to the peculiar characteristics of the structure of proteins—the sequence of the amino acid formation and amino acid arrangement in space forming geometric forms.

The recent advances have been so great that people have begun thinking of the possibilities during the next decade or so, to have synthetic substances which would have quite a good characteristic of these proteins.

During the recent past, work has been going on adventurously towards the understanding of the life process itself by synthesising protein-like substances in the laboratory.



Proteins have particular characteristics. They sustain life and serve as food to our people. We can, at the discussion, think of methods for the future development of protein industry in the country not only in regard to food, but also for extremely important industries like production of plastics. In future, it would be useful to take out the sub-branches of this type from different angles in smaller groups in order to get more knowledge. A large number of papers and abstracts have been received which have a bearing on the chemical, biological, nutritional, technological and therapeutic aspects. Protein chemistry is an exceedingly important one and I take this opportunity to thank the CSIR for according approval for holding the symposium here and on a subject of such great basic and practical importance. I must also thank the band of devoted workers who have contributed papers to the symposium and others who have laboured to make it a success.

*Prof. M. S. Thacker*

Shri Narielwala, Dr Guha, Dr Subrahmanyam, Dr Sreenivasan, Ladies and Gentlemen,

I consider it a great privilege to be amidst you this afternoon and to inaugurate the Symposium on Proteins organised by the Chemical Research Committee of CSIR and the Society of Biological Chemists. Dr Subrahmanyam and Dr Guha have spoken to you already on proteins and it would be presumptuous on my part to think that I can add to what has been said very well by them. In the technical session, that will follow, various aspects of proteins will receive expert treatment at the hands of the fellow-scientists attending the Symposium.

But may I take this opportunity to think, aloud with you this afternoon on certain general aspects pertaining to the field of science and scientific research in India? I may, for the moment, make a reference to what Dr Subrahmanyam mentioned about the recent meeting in London of the Heads of scientific organizations of the countries of the Commonwealth where I had the privilege of being elected as its Chairman for a period of three years from the beginning of the next year. In this meeting were discussed not only matters relating to the administration of scientific research but also evaluation of research programmes in the countries of the Commonwealth in a free and frank way, without any mental reservation. The Heads of several scientific research organizations of the Commonwealth countries spoke with warm appreciation about scientific research in the Indian national laboratories, universities and other institutions during the last ten years. It is a matter of gratification that, in relation to the advanced countries of the Commonwealth, India has more than acquitted herself in the field of scientific research. There is thus a picture of scientific research in India painted outside as progressing dynamically.

I had this morning the opportunity to preside over the Executive Council of this Institute and discuss frankly its achievements. One thing emerged; here is a laboratory engaged in one of the disciplines—a discipline which is of the greatest importance to the country, *viz.*, Food, and, in its various activities, it has done a good measure of useful work which could go outside and help in the solution of the food problems facing the country.

We have several industrialists and other members of the Executive Council seeking assistance from this laboratory. I would like to say that they are willing, at all times,



to extend their co-operation; but, at times, things do come to a stand-still or the response is not forthcoming in an adequate measure. Here is a problem which I have not been able to analyse completely and I am passing on the problem here to a gathering of scientists and industrialists so as to help us in that analysis. Why is it that the work of the laboratories is not finding application in the development and strengthening of economy of the country? Every colleague of mine in this laboratory wishes to see at times the practical application of the scientific research on which he has worked successfully. There seems to be frustration among some of my colleagues. But I would like to say that the apathy which existed years back towards scientific research does not now exist to that extent and there has been a complete change. The country has realised, and realised fully, the importance of scientific and technological research to develop on modern lines her economy for the betterment of the whole community.

Here I wish to make an appeal that in the Third Five Year Plan, in the planning of the integrated development in the fields of agriculture, industry—heavy or chemical—transportation, education, etc., the scientific research must receive the same priority if not a higher priority. If scientific research is financed even to the extent of one per cent of the total outlay of the Third Five Year Plan, the progress of science in India will doubtless attain the necessary momentum.

Mr Chairman, I have left proteins this afternoon, but to us scientists, this is a matter of proteins in our work.

In the matter of the present symposium on Proteins, I have no doubt that the research workers assembled here will discuss various aspects of proteins including protein malnutrition on which considerable work is being done in the country. I would like to make one observation that, immediately at the end of the Symposium, a number of my colleagues are proceeding to the U.S.A. to attend an International Conference on Protein Malnutrition. I hope the deliberations of the symposium will give them sufficient material to take with them and present at the Conference.

This symposium has been organised by the Chemical Research Committee of which Dr Guha is the Chairman and the Society of Biological Chemists headed by Dr Khanolkar. Thanks are due to them and also to Dr Subrahmanyam and his colleagues, who have worked hard to make the function really worthwhile. I wish you all success and have pleasure in inaugurating the symposium on proteins.

*Shri P. A. Narielwala*

Prof. Thacker, Dr Subrahmanyam, Shri Vasudeva Rao, Ladies and Gentlemen,

When I came here yesterday, I had no idea that I would be asked to preside over the symposium. In fact, I came as a layman and an observer. We have heard Dr Subrahmanyam and Dr Guha, telling us something about proteins, which for a layman like myself, is of fascinating interest.

A few years ago, the United States set up an International Committee realising the need for protein or supplementation of proteins to people's dietaries in the countries of Asia and Africa, which are generally undernourished and underfed. It is gratifying to note that on the International Committee, from its very inception, Indian scientists



have found a place, because it has been recognised that our scientists all over the country have done some valuable work on proteins. In this laboratory also, some pioneering work on the isolation of proteins from vegetable sources has been done. It was of interest to us because if we could isolate protein in a satisfactory economical manner, it would open for us, in this country, a vast field of developing a protein industry which will help feed our undernourished and underfed population, particularly of the age group between 2-8 who are our future hopes. The future of the country depends on how much more of vegetable protein we can add to the inadequate diets of the masses and to what extent we can offer help for building up physical development.

I have been associated with the work of some of the national laboratories in the countries which are tackling these problems. These problems are right before most of us in the industry also. There should be a two-way traffic between national laboratories, universities, research institutions, and the industry. Industry is not fully aware of the work that is being done in many of our national laboratories and institutions and the fault does not entirely lie with the Industry. The laboratories should, therefore, take special care to see that their work is well known, at least in these circles where it has the potentiality of being utilized.

*Dr A. Sreenivasan*

I have a very pleasant duty to perform in offering thanks to everyone. A symposium of this kind on a restricted but topical subject is of extreme importance for the ever expanding knowledge of the frontiers of science and technology. It enables one to learn, at a glance, the highlights of progress being made and the trends of future research. It relieves the burden of plodding through the voluminous literature and brings together men from academic, industrial, governmental and other spheres into close intellectual contact during the next two days. The formal and informal meetings, I am sure, are bound to give birth to new ideas for the future. The scientist will get back to his work greatly encouraged and the industrialist greatly vitalised.

We, in this Institute, are particularly happy at the choice of the venue, not only because of the current interest of the Institute in the field of protein malnutrition but also because of our own efforts along practical lines in the amelioration of protein malnutrition of the people at large. We hope that the industry will follow up on our efforts.

Our first thanks go to the CSIR and its CRC with its dynamic Chairman, Dr Guha and to the Society of Biological Chemists for having made the symposium possible. I should thank Shri Narielwala for having given us his kind encouragement. I should thank Shri Vasudeva Rao for having made it possible for us to listen to the stimulating speech of the Chief Minister and I should also thank all the participants and contributors to the symposium for having readily responded to the invitation. I am also thankful to my colleagues who have helped a great deal in the organisation.

The symposium is bound to be a success in regard to the papers that are going to be presented. I would say that this is due to the excellent response that has been received and I express the hope that the next two days will be of useful purpose to all of us.







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# CONTENTS

## I. CHEMISTRY

	PAGE
THE ARCHIBALD METHOD OF MOLECULAR WEIGHT DETERMINATION WITH THE ULTRACENTRIFUGE By M. S. Narasinga Rao	1
FRACTIONATION, ISOLATION AND ELECTROPHORETIC CHARACTERIZATION OF RED GRAM GLOBULINS By Saroj Tawde and H. R. Cama	8
HETEROGENEITY OF BUFFALO HAEMOGLOBIN By G. J. S. Rao and H. R. Cama	15
ISOLATION OF AN ELECTROPHORETICALLY HOMOGENEOUS $\alpha_1$ -MUCOPROTEIN FROM SHEEP PLASMA By B. R. Das	20
USE OF ION-EXCHANGERS IN THE SEPARATION OF PROTEINS OF COBRA VENOM By Vijaya N. Waknis, R. W. P. Master and S. S. Rao	25
STUDY OF PROTEIN-PROTEIN INTERACTIONS IN SERUM UNDER THE INFLUENCE OF HEAT By Surbala L. Shah and S. S. Rao	31
EFFECT OF ACIDS ON PROTEINS By K. A. Abraham and P. M. Bhargava	37
REACTIONS BETWEEN FORMALDEHYDE AND PEPTONE By V. Subba Rao	47
COMPARATIVE STUDY OF ALKALI AND SWAN'S REAGENT IN THE EXTRACTION OF PROTEINS FROM PLANT MATERIALS By C. R. Krishna Murti	50
THE USE OF HEXAMETAPHOSPHATE IN THE ISOLATION OF BIOLOGICALLY ACTIVE BASIC PROTEINS By O. P. Shukla and C. R. Krishna Murti	55
ESTIMATION OF IODINE IN PROTEIN-IODINE COMPLEXES By Rabindra N. Bhattacharya and S. K. Dutta	59
STUDIES ON 'IODOTHYRINS' By E. Raghupathy, P. V. Narasimha Murthy and P. S. Sarma	61
A SIMPLE PROCEDURE FOR SEPARATING PROTEINS IN THE ISOLATION OF PENTOSE-NUCLEIC ACID AND DESOXYPENTOSE-NUCLEIC ACID FROM MAMMALIAN TISSUES By S. K. Dutta and S. Pal	67

## II. BIOCHEMISTRY

VITAMIN B <sub>12</sub> AND PROTEIN BIOSYNTHESIS By Ranjan Bhatt, S. R. Wagle and B. Connor Johnson	73
ROLE OF VITAMIN B <sub>12</sub> IN PROTEIN SYNTHESIS BY <i>ESCHERICHIA COLI</i> By C. R. Krishna Murti	78
INTERDEPENDENCE OF RIBONUCLEIC ACID AND PROTEIN SYNTHESIS IN <i>SACCHAROMYCES CEREVISIAE</i> By D. S. Pradhan, D. V. Rege and A. Sreenivasan	83
THE RELATIONSHIP OF INDUCED CATALASE SYNTHESIS TO RIBONUCLEIC ACID METABOLISM IN YEAST By C. Bhuvaneswaran, D. V. Rege and A. Sreenivasan	90
EFFECT OF STREPTOMYCIN ON PROTEIN SYNTHESIS IN <i>ESCHERICHIA COLI</i> By Nitya Anand and B. D. Davis	100



DIETARY PROTEIN QUALITY AND VITAMIN A UTILIZATION—By G. C. Esh and R. K. Bhattacharya	107
HEMATOPOIESIS AND NITROGEN METABOLISM IN THE RIBOFLAVIN DEFICIENT RAT By S. Mookerjea	114
PROTEIN METABOLISM IN SCURVY By Sachchidananda Banerjee	124
INFLUENCE OF DIETARY PROTEIN LEVELS ON THE VITAMIN C STATUS OF THE RAT By H. N. Bhagavan, M. V. L. Rao and M. Srinivasan	129
A STUDY OF THE EFFECTS OF VARIATIONS IN PROTEIN QUALITY AND PROTEIN LEVEL ON GROWTH AND BODY COMPOSITION OF RATS By Urmila Marfatia, T. B. Rao and A. Sreenivasan	133
EFFECTS OF [PROTEIN PER CALORIE RATIO, DIETARY FAT LEVEL AND B VITAMINS ON PROTEINS AND CALORIE UTILIZATION IN THE GROWING RAT By Prema Fatterpaker, W. V. Lavate, A. G. Mulgaonkar and A. Sreenivasan	140
A STUDY OF THE EFFECT OF VARIATION IN DIETARY FAT LEVELS, PROTEIN QUALITY AND B VITAMINS SUPPLEMENTATION, ON LIPID COMPOSITION AND DISTRIBUTION IN BLOOD AND LIVER IN RATS—By Urmila Marfatia, T. B. Rao and A. Sreenivasan	147
THE RELATIONSHIP OF DIETARY B VITAMINS AND PROTEINS TO REGENERA- TION OF TISSUE CONSTITUENTS IN THE PROTEIN DEPLETED RAT By D. S. Wagle and A. Sreenivasan	154
SUPRARENAL CYTOPLASMIC PROTEIN PATTERN IN NORMAL AND IN COLD STRESSED RATS By B. M. Roichaudhuri, Anubha Chowdhury and P. B. Sen	168
COMPARATIVE TRANSAMINATION CAPACITY ON COLD EXPOSURE—By D. P. Sadhu	172
INTERRELATIONSHIP BETWEEN ADRENOCORTICOTROPHIC AND MELANOCYTE STIMULATING HORMONES By J. N. Karkun and B. Mukerji	175
EFFECTS OF PROTEINS AND AMINO ACIDS ON BLOOD SUGAR LEVELS AND GLUCOSE TOLERANCE By M. V. L. Rao, J. Meena Rao and M. Srinivasan	181
METABOLISM OF MUCOPOLYSACCHARIDES DURING CONNECTIVE TISSUE FORMATION—By A. Guha, A. S. Balasubramaniam, D. K. Basu, T. N. Pattabiraman and B. K. Bachhawat	187
IMMUNOCHEMICAL STUDIES ON THE PROTEINS OF <i>VIBRIO CHOLERAE</i> By S. K. Sharma, K. D. Pant and D. L. Shrivastava	194
STUDIES ON THE CHARACTERIZATION AND THE MECHANISM OF FORMATION OF AN EXO-ENZYME PROTEIN (LECITHINASE) IN <i>VIBRIO EL TOR</i> —By S. Mitra and G. C. Chatterjee	197
ALANINE DEHYDROGENASE IN MYCOBACTERIA—By N.A.N. Rao, T. Ramakrishnan and M. Sirsi	204
MICROBIOLOGICAL EVALUATION OF HEART EXTRACTS—By V. G. Pradhan and J. V. Bhat	212
THE ANTIGENICITY OF HUMAN SPERMATOZOA AND ITS SIGNIFICANCE By Shanta S. Rao, Katy K. Sadri and Anil R. Sheth	218
OCCURRENCE OF FERRIC ION REQUIRING ALKALINE PHOSPHATASE IN PLANTS By N. Appaji Rao, H. R. Cama, S. A. Kumar and C. S. Vaidyanathan	226



A NATURALLY OCCURRING INHIBITOR OF GLUTAMINE SYNTHESIS PRESENT IN <i>PONGAMIA</i> GALLS	By (Miss) N. K. Sukanya and C. S. Vaidyanathan	232
THE INFLUENCE OF CHELATING AGENTS ON THE TRANSPORT OF COBALT BY BLOOD PROTEINS	By V. M. Sivaramakrishnan, T. N. Sekhara Varma and M. R. Lakshmanan	239
PROTEIN METABOLISM IN UREMIA	By C. J. Mehta	244
EFFECT OF IRON CHLOROSIS ON PROTEIN FRACTIONS OF CORN ( <i>ZEA MAYS</i> ) LEAF TISSUE	By N. G. Perur	248

### III. TECHNOLOGY

LEAF PROTEIN TECHNOLOGY	By P. R. Chakravorty and B. C. Guha	257
PRODUCTION OF PROTEIN ISOLATE FROM GROUNDNUT (PEANUT)—By D.S. Bhatia, S. S. Kalbag, N. Subramanian, K. E. Eapen, B. S. Ramachandra, K. Anantharaman, K. K. Gopalan, V. Subrahmanian, B. R. Ramanna, M. N. Narayana, K. Narayana, A. Sreenivasan and V. Subrahmanyam		260
STUDY OF SOME OF THE OPERATIONS IN THE INTEGRATED PROCESS FOR GROUNDNUTS	By K. E. Eapen and S. S. Kalbag	266
TECHNOLOGY OF PROTEIN-RICH NUTRO FOODS—By G. S. Bains, D. S. Bhatia and V. Subrahmanyam		270
STUDIES ON THE NUTRITIVE VALUE OF PROCESSED PROTEIN FOODS BASED ON OIL SEED MEALS FORTIFIED WITH VITAMINS AND MINERALS—By M. Narayana Rao, Kantha Joseph, R. Rajagopalan and M. Swaminathan		276
DEVELOPMENT OF PRECOOKED BALANCED PROTEIN FOOD SUITABLE FOR WEANED INFANTS—By M. Narayana Rao, Kantha Joseph, M. Swaminathan and V. Subrahmanyam		286
THE NUTRITIONAL VALUE OF FISH FLOUR—By A. B. Morrison and J. A. Campbell		290
SOME PROTEIN-RICH PROCESSED FOODS FROM FISH—By M. N. Moorjani and N. L. Lahiry		296
PREPARATION OF CHEDDAR AND PROCESSED CHEESE USING THE MILK CLOTTING ENZYME FROM <i>FICUS CARICA L</i> —By M. A. Krishnaswamy and D. S. Johar		302
PREPARATION OF PREDIGESTED PROTEIN FOODS—By Bhagwan S. Lulla, Dayal Singh Johar and V. Subrahmanyam		307
PRODUCTS OF PROTEIN HYDROLYSIS FROM FISH—By D. P. Sen and N. L. Lahiry		312
PROTEIN HYDROLYSATES FROM INDIGENOUS SOURCES—By M. K. Rastogi, C. Singh and C. R. Krishna Murti		318
PROTEIN DENATURATION OF FISH TISSUE ON STORAGE IN ICE—By D. Roy Chaudhuri and A. N. Bose		326
EFFECT OF PROCESSING ON THE SOLUBILITY OF PROTEINS IN SOME STRAINED BABY FOODS—By S. Ranganna, A. M. Nanjunda Swamy and G. S. Siddappa		329
SOME OBSERVATIONS ON THE PREPARATION OF MEAT EXTRACT, MEAT SOUP CUBES AND CHICKEN ESSENCE	By B. R. Baliga, N. V. Sripathy, N. V. R. Iyengar and N. L. Lahiry	335



#### IV. NUTRITION

NUTRITIVE EVALUATION OF VEGETABLE PROTEINS	By S. V. Phansalkar	345
BIOLOGICAL VALUE AND ESSENTIAL AMINO ACID COMPOSITION OF THE PROTEINS OF SOME PULSES	By Sachchidananda Banerjee	355
HIGH AND LOW PROTEIN BENGAL GRAM: NITROGEN DISTRIBUTION AND NUTRITIONAL ASSESSMENT	By G. C. Esh and T. S. De	357
NUTRITIONAL EVALUATION OF VEGETABLE PROTEIN ISOLATES AND THEIR FORMULATIONS—By K. Anantharaman, N. Subramanian, K. K. Gopalan and D. S. Bhatia		363
THE NUTRITIVE VALUE OF PROTEIN BLENDS SIMILAR TO FAO REFERENCE PROTEIN PATTERN IN AMINO ACID COMPOSITION—By Kantha Joseph, M. Narayana Rao, M. Swaminathan, K. Indiramma and V. Subrahmanyam		369
STUDIES IN NUTRITIVE VALUE OF DOUBLE BEAN ( <i>VICIA FABA</i> , MOENCH)	By A. N. Inamdar and Kamala Sohoni	375
CLINICAL TRIALS WITH VEGETABLE PROTEIN FOODS—By P. S. Venkatachalam and S. G. Srikantia		381
TREATMENT OF PROTEIN MALNUTRITION IN CHILDREN WITH A VEGETABLE PROTEIN FOOD	By G. Purshowtham Rao	389
TREATMENT OF PROTEIN MALNUTRITION IN CHILDREN USING BLENDS OF ISOLATED VEGETABLE PROTEINS, CASEIN AND SKIM MILK POWDER	By R. K. Bhagavan, T. R. Doraiswamy, N. Subramanian, K. Anantaraman and M. Narayana Rao	393
PROTEIN HYDROLYSATE IN RELATION TO HAEMOPOIESIS AND PROTEIN REGENERATION	By A. N. Bose and P. K. Guha	397
BIOCHEMICAL STUDIES IN PROTEIN MALNUTRITION—By J. Nagchaudhuri, S. P. Ghosal, K. K. Chatterjee and K. C. Chaudhuri		405
INDIAN MULTIPURPOSE FOOD—A LOW COST PROTEIN SUPPLEMENT TO THE INDIAN DIETARY	By Radha Karnad	415
THE PLACE OF A COMPOSITE PROTEIN FOOD IN SCHOOL LUNCH PROGRAMMES	By P. Geervani	421
INFLUENCE OF VARIETY AND CULTURAL CONDITIONS ON THE PROTEIN CONTENT IN FOODSTUFFS	By N. B. Das	431
NITROGENOUS SUBSTANCES IN PASSION FRUIT ( <i>PASSIFLORA EDULIS</i> SIMS.) WASTE	By R. Susheela, J. S. Pruthi and Girdhari Lal	435
AMINO ACID COMPOSITION OF LARVAE OF THE POTATO MOTH ( <i>GNORIMOS-CHOMA OPERCULELLA</i> ZELL.) AND THEIR NUTRITIVE VALUE FOR REARING PARASITES	By S. P. Bhatnagar	439
AUTHOR INDEX		441
SUBJECT INDEX		449



# I. CHEMISTRY

*Moderator:*

PROF. P. S. SARMA







# THE ARCHIBALD METHOD OF MOLECULAR WEIGHT DETERMINATION WITH THE ULTRACENTRIFUGE

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The well-known methods for the determination of the molecular weight of a macromolecular substance with the ultracentrifuge are the sedimentation velocity and sedimentation equilibrium methods, developed by Svedberg<sup>1</sup>. In recent years another procedure has been described by Klainer and Kegeles<sup>2</sup>. This is based on the suggestions of Archibald<sup>3</sup> that the equation employed in the equilibrium method is valid at the top and the bottom meniscus of the liquid column in the cell, even when equilibrium is not reached. This method, generally known as the Archibald method, combines the advantages of the velocity and equilibrium methods in so far as the diffusion coefficient of the solute is not required to calculate the molecular weight and further, the experiment is essentially of short duration.

It has been applied to determine the molecular weights of homogeneous proteins such as bovine plasma albumin<sup>2,4</sup>, of mixtures of proteins<sup>5</sup>, of polydisperse synthetic polymers<sup>6</sup> and of proteins which undergo reversible association-dissociation reactions<sup>7,8</sup>. The latter is of considerable interest in biochemistry because a number of proteins such as insulin, chymotrypsin, papain, etc., possesses this property.

The purpose of this paper is to describe the application of the method to such systems. A few results of a study of the polymerization of  $\alpha$ -chymotrypsin in phosphate buffer solutions of different ionic strength have been included to illustrate the method. The polymerization of  $\alpha$ -chymotrypsin (phosphate buffer, 0.20 ionic strength and pH 6.2) has been reported by Rao and Kegeles<sup>8</sup>.

## Experimental

The  $\alpha$ -chymotrypsin was a Worthington Biochemical Corporation product, lot CDI 537.40. Molecular weight measurements were made in phosphate buffer solutions of 0.10 ionic strength (pH 6.9) and 0.05 ionic strength (pH 7.4). Protein dissolved in the buffer was dialysed against a large volume of the buffer solution in the cold for about 12 hr.  $\alpha$ -chymotrypsin is isoelectric in these solutions and hence the effects of the non-ideality of the solution are expected to be minimum in this condition. Isoelectric points were determined by electrophoretic mobility measurements with a Tiselius Electrophoresis Apparatus (Perkin-Elmer Model 38). The variation of the isoelectric point with phosphate ion concentration may be attributed to the binding of these ions by the protein.

The protein concentrations were determined by measuring the ultraviolet absorption at 282 m $\mu$  and using the value of 2.07 for the specific absorption co-efficient<sup>8</sup>. For the calculation of molecular weight a value of 0.736 was used for the partial specific volume of  $\alpha$ -chymotrypsin<sup>9</sup>. The experiments were done at room temperature.

Molecular weight determinations were done with a Spinco Model E Ultracentrifuge. The experimental technique and the method of calculation proposed by Klainer and Kegeles<sup>2</sup> were followed. A brief description of the method is given here.



The equation for the calculation of molecular weight,  $M$ , is

$$M = \frac{RT \, dc/dx}{(1-V\rho) \, \omega^2 x C} \dots\dots\dots(1)$$

where  $R$  is the gas constant,  $T$  the absolute temperature,  $V$  the partial specific volume of the protein,  $\rho$  the density of the solution,  $\omega$  the angular velocity,  $x$  the radius of rotation corresponding to the top or the bottom of the liquid column,  $dc/dx$  the concentration gradient and  $C$  the total solute concentration. It may be pointed out here that this equation, which is the same as the sedimentation equilibrium equation, is valid in the Archibald method at only two planes, namely, the top and the bottom of the liquid column in the cell and nowhere else.

The cylindrical lens Schlieren optical system used in the Model E Ultracentrifuge registers directly a quantity proportional to the concentration gradient,  $dc/dx$ . The corresponding concentration,  $C$ , is obtained in terms of the original solute concentration,  $C_0$ , with the following equation<sup>2</sup>.

$$\left. \begin{aligned} C_{x_0} &= C_0 - \frac{1}{X_0^2} \sum_{x_0}^x x^2 (dc/dx) \cdot dx \\ C_{x_b} &= C_0 + \frac{1}{X_b^2} \sum_x^{x_b} x^2 (dc/dx) \cdot dx \end{aligned} \right\} \dots\dots\dots(2)$$

where  $X_0$  and  $X_b$  are the radii corresponding to the top and the bottom respectively and  $X$  the radius of any plane in the 'plateau region' where  $dc/dx = 0$ .

The synthetic boundary cell described by Kegeles<sup>10</sup> is used for the determination of  $C_0$ . The two side holes of the cell are filled with the solvent (phosphate buffer solution) and the centre of the cell with the protein solution. The solvent layers itself on the solution without convective mixing when the cell is centrifuged at a low speed. The resulting boundary is photographed. A typical boundary pattern is given in Fig. 1.

For the next part of the experiment a standard 4° sector cell is used. First, about 0.1 c.c. of a dense, inert liquid such as carbon tetrachloride is introduced and then about 0.7 c.c. of the protein solution. The carbon tetrachloride creates a visible, false bottom and obviates certain optical difficulties associated with the location of the true cell bottom. The cell is then centrifuged at a known, constant r.p.m. and the Schlieren patterns are photographed at regular intervals of time. The experiment is usually done for 1-2 hours. The temperature of the cell is measured both at the beginning and the end of a run. A representative Schlieren pattern is given in Fig. 2. In Figures 1 & 2, the vertical readings correspond to  $dc/dx$  values and the horizontal readings to  $x$  values.

The photographic plate is then measured with a two dimensional micro-comparator. With the concentration boundary,  $dc/dx$  values are read at intervals of, say, 0.025 c.m., and the sum of these values multiplied by 0.025 gives a numerical value proportional to  $C_0$ . With the other pattern,  $dc/dx$  values are read at 0.025 c.m. intervals starting from the top meniscus to the 'plateau' region. Similar readings are taken from the 'plateau' region to the bottom meniscus. The distance of the midpoint of the cell to the axis of rotation in the ultracentrifuge is exactly 6.5000 c.m. Knowing the magnification factor of the optical system the true radius corresponding to the top and the bottom of the liquid column



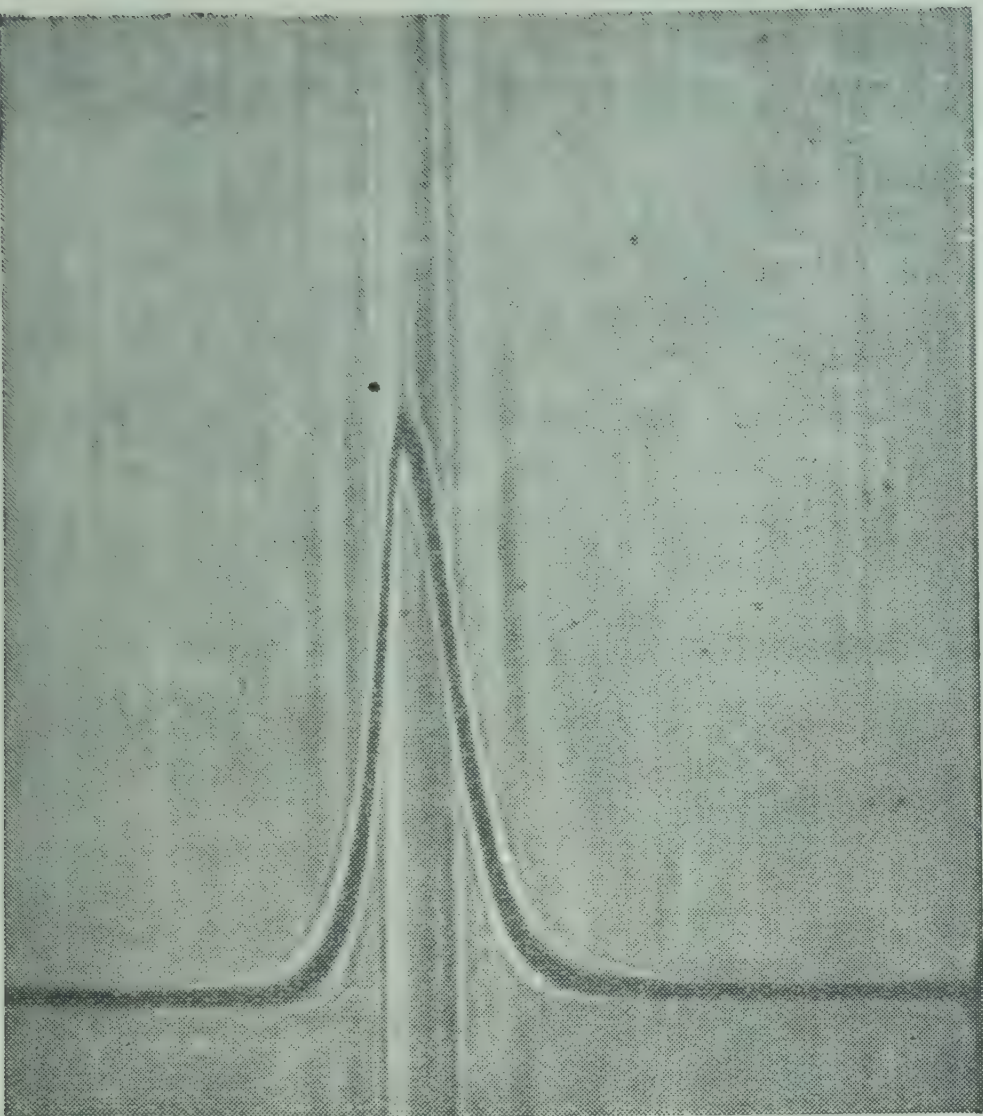


FIG. 1. A representative concentration boundary pattern.

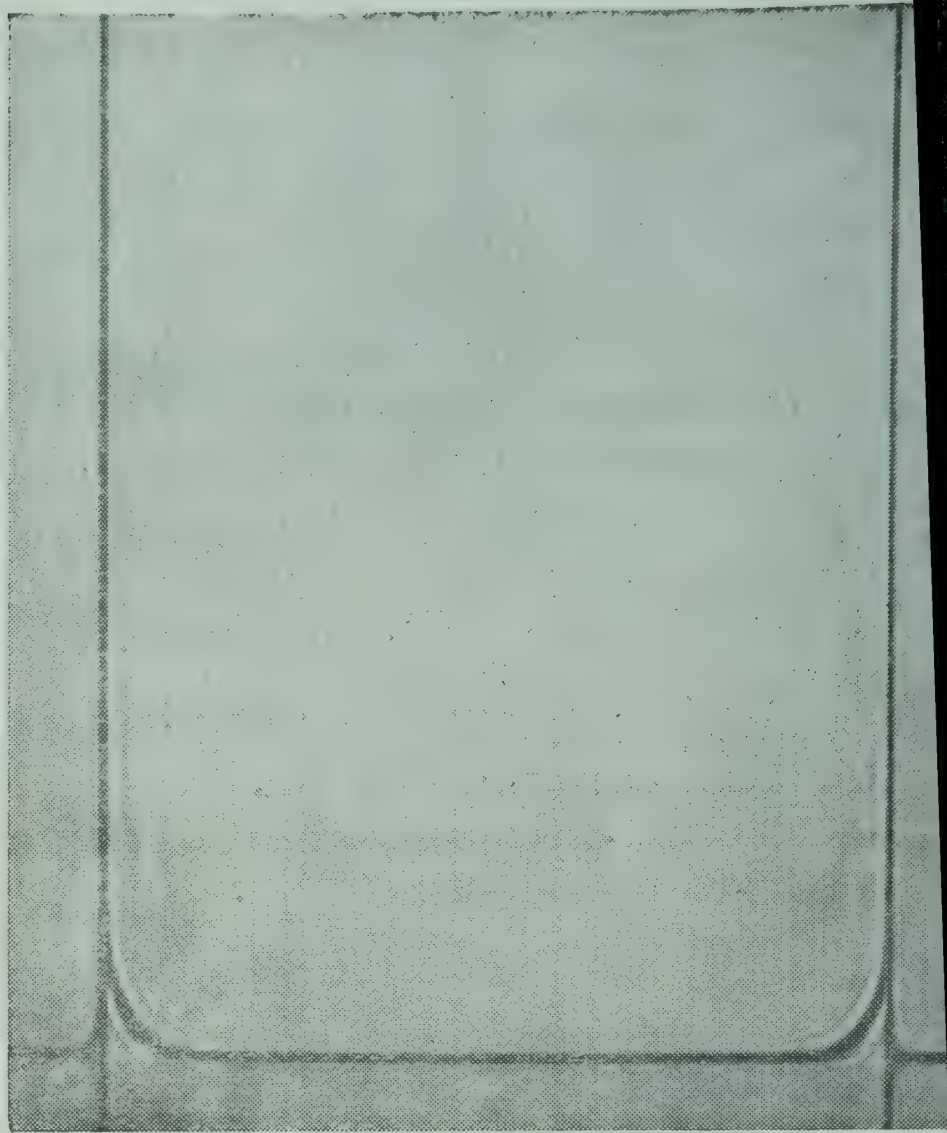


FIG. 2. A representative ultracentrifuge pattern in the Archibald run.

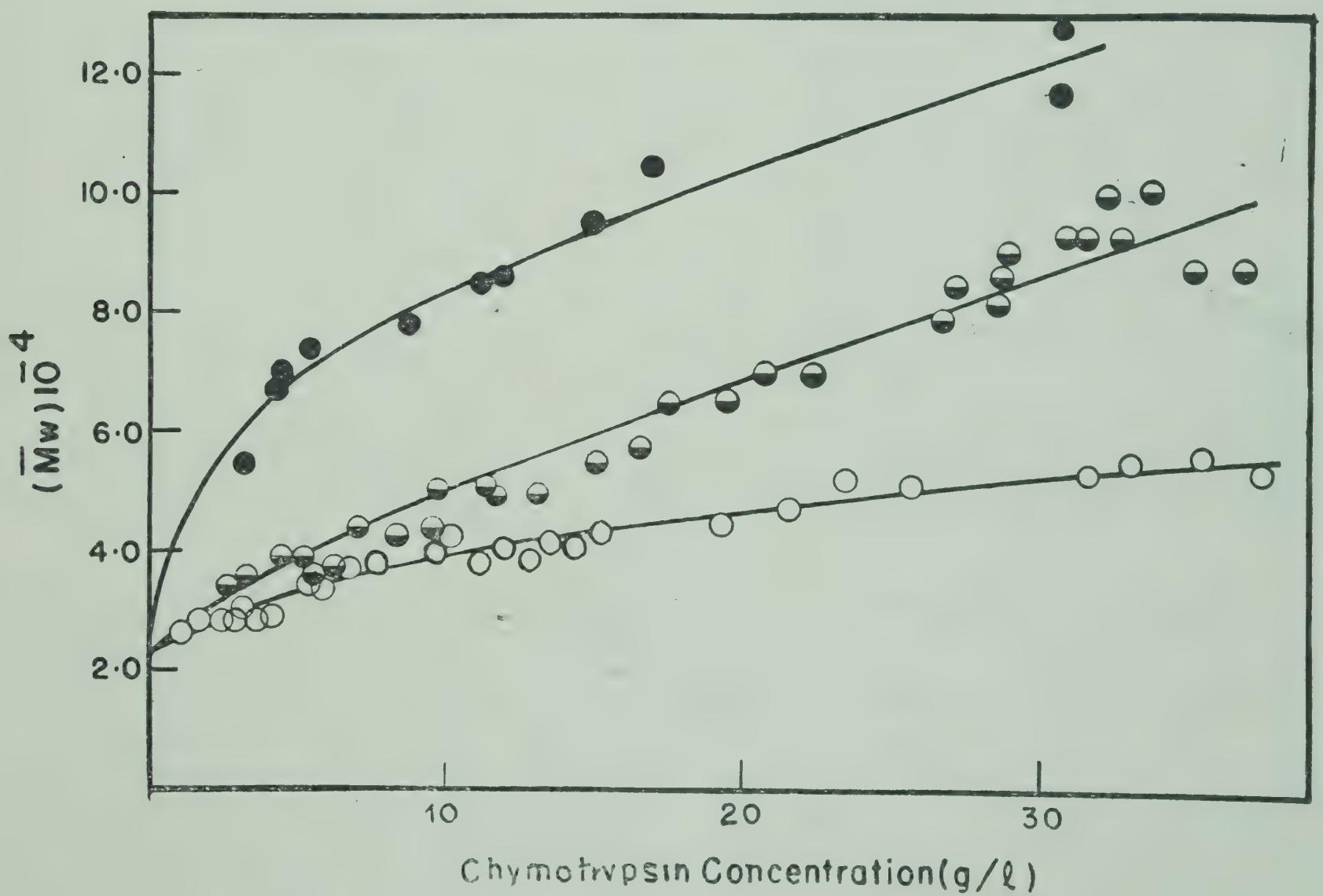


FIG. 3. Experimental Weight-Average Molecular weight *versus* chymotrypsin concentration.

- phosphate buffer, pH 6.2 and  $\mu=0.20$
- ◐ phosphate buffer, pH 6.9 and  $\mu=0.10$
- phosphate buffer, pH 7.4 and  $\mu=0.05$



and also to other points where  $dc/dx$  values are read can be calculated. The summation on the right hand side of equation (2) can be computed with these values. At either of the two radii  $X_0$  or  $X_b$ ,  $dc/dx$  value cannot be read with precision directly on the micro-comparator because of optical distortions and the data is therefore extrapolated to these radii. The data needed to calculate  $M$  from equation (1) are thus obtained.

The extrapolation procedure is a disadvantage of the method, as it can lead to considerable uncertainty in steep gradients. The experiments are therefore done at moderate speeds to avoid setting up large gradients. This has an added advantage in case of associating-dissociating protein systems.  $C_{x_0}$  and  $C_{x_b}$  will not be very far from  $C_0$  and thus, the chemical equilibrium is not greatly disturbed. A requisite of Klainer-Kegeles technique is that there should always be a 'plateau' region. This is possible in experiments of short duration. If ultracentrifugation is continued for a long time, the 'plateau' region disappears and equation (2) is not then valid.

## Results and Discussion

The weight-average molecular weight of  $\alpha$ -chymotrypsin at various protein concentrations in phosphate buffer solutions of ionic strengths ( $\mu$ ) 0.10 & 0.05 determined by this technique are given in Fig. 3. The data at  $\mu = 0.20$  are taken from the author's

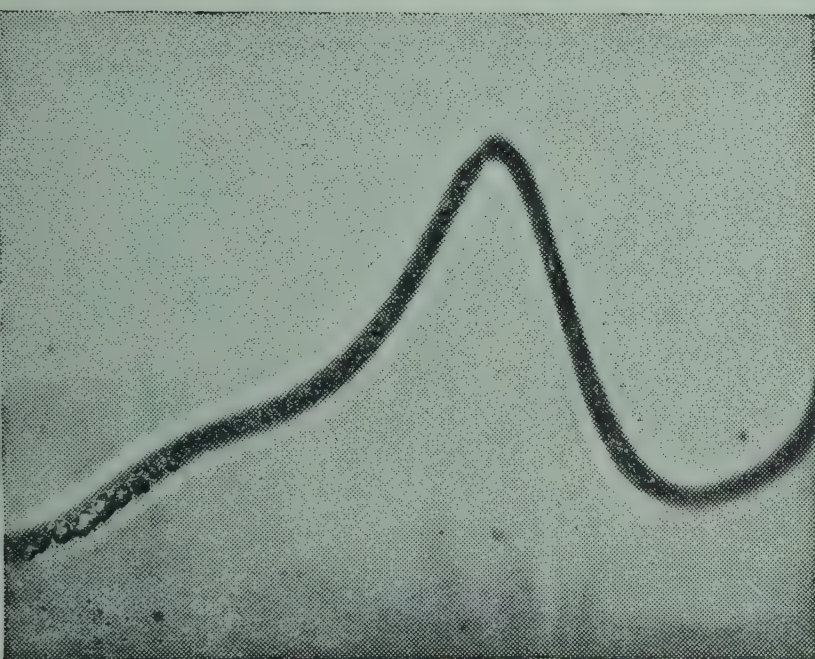


FIG. 4 A

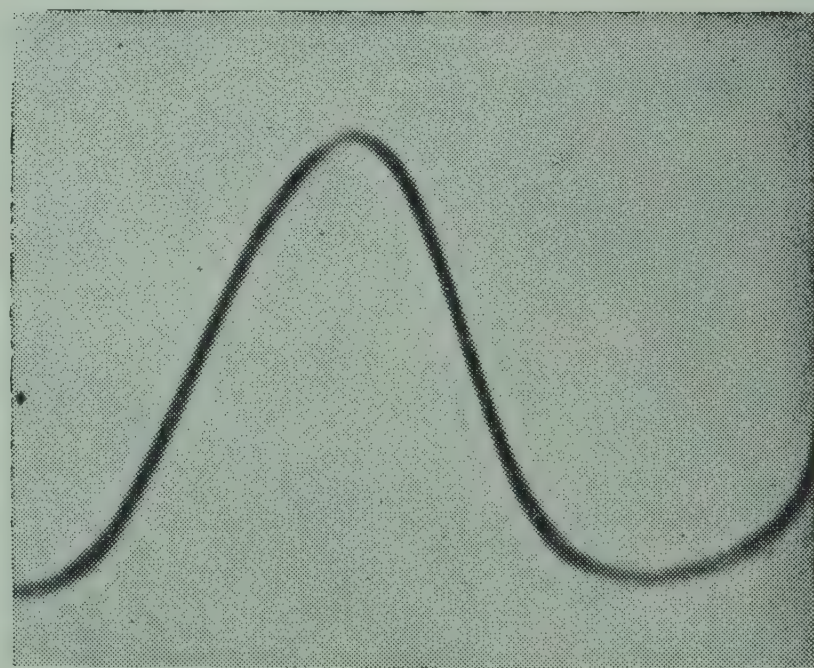


FIG. 4 B

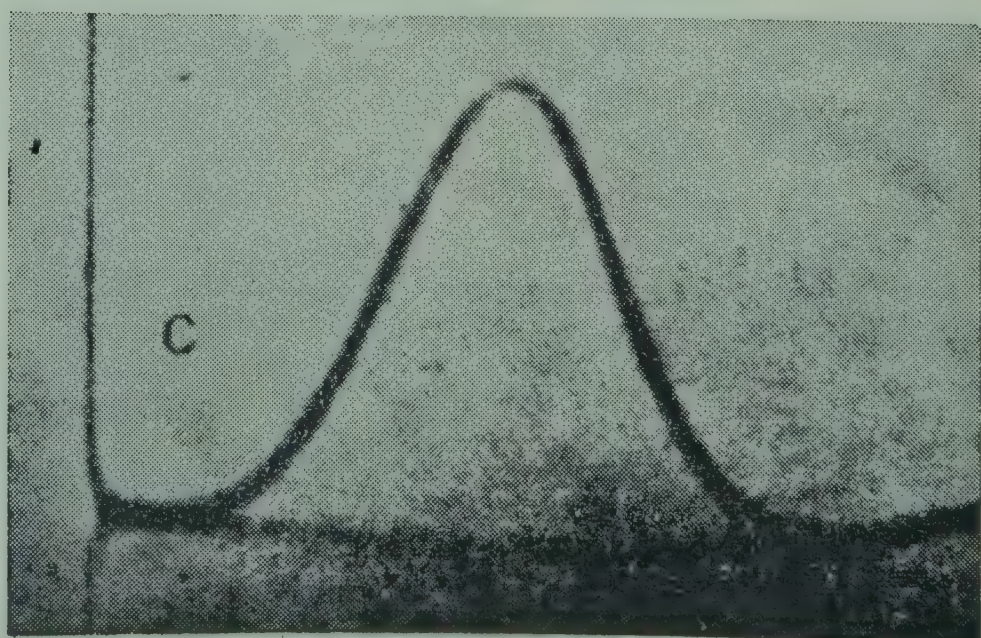


FIG. 4. Sedimentation velocity patterns of chymotrypsin.

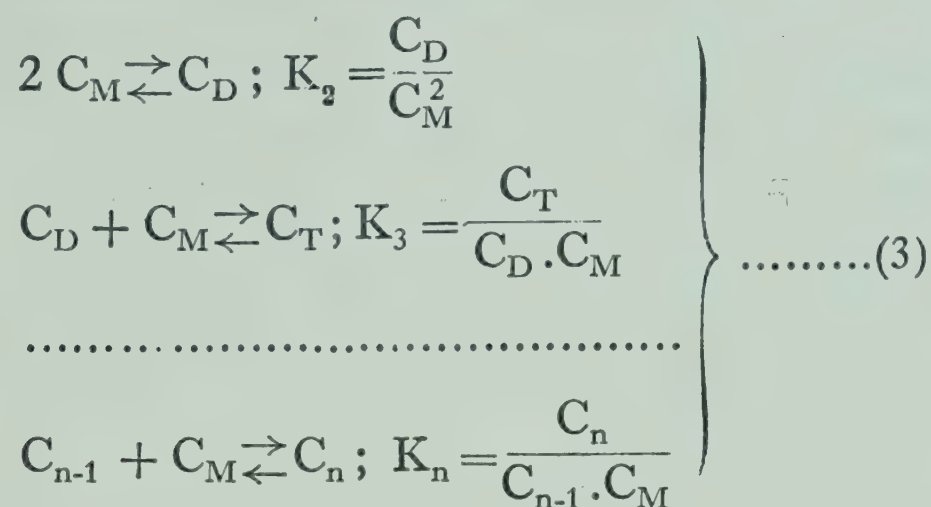
- A. Protein concn., 2.8%; phosphate buffer, pH 7.4 and  $\mu=0.05$ ; 45 min. of centrifugation at 59,780 r.p.m.
- B. Protein concn., 3.4%; phosphate buffer, pH 6.9 and  $\mu=0.10$ ; 60 min. of centrifugation at 59,780 r.p.m.
- C. Protein concn., 1.89%; phosphate buffer, pH 6.2 and  $\mu=0.20$ ; 120 min. of centrifugation at 59,780 r.p.m.

Sedimentation proceeds from left to right.



published results<sup>8</sup>. It is seen that the extent of polymerization increases inversely as the ionic strength of the phosphate buffer. For example, at a protein concentration level of 10 g./l., the weight-average molecular weight is  $(40.0) 10^3$  at  $\mu = 0.20$ ,  $(50.0) 10^3$  at  $\mu = 0.10$  and  $(83.0) 10^3$  at  $\mu = 0.05$ . These observations are in qualitative agreement with the sedimentation velocity results of Massey *et al.*<sup>11</sup>. The data extrapolated to  $C = 0$  lead to a value of 23,000 for the monomer molecular weight. This is in good agreement with the value obtained by other methods<sup>12,13</sup>.

To get an idea of the type of polymers formed, the data can be analysed in terms of equilibria for the stepwise formation of polymers, as follows:



where,  $C_M$ ,  $C_D$ ,  $C_T$ ,  $\dots C_n$  are the weight-concentrations of the monomer, dimer, trimer  $\dots$   $n$ -mer respectively in the equilibrium mixture and  $K_2$ ,  $K_3$ ,  $\dots K_n$  are the equilibrium constants for the formation of dimer, trimer,  $\dots$   $n$ -mer. The weight-average molecular weight,  $\bar{M}_w$ , at any protein concentration,  $C$ , is given by<sup>8</sup>:

$$\bar{M}_w = \frac{C_M M_1 + 2K_2 C_M^2 M_1 + 3K_2 K_3 C_M^3 M_1 + \dots + nK_1 \dots K_n C_M^n M_1}{C} \dots (4)$$

where  $M_1$  is the monomer molecular weight. The value of  $C_M$  as a function of  $C$  can be obtained by the graphical integration method proposed by Steiner<sup>14</sup>. Then, from the limiting slope at  $C_M = 0$  of  $(\bar{C}M_w/C_M M_1)$  as a function of  $C_M$ ,  $K_2$  can be obtained. Next,

$\left( \frac{\bar{C}M_w}{C_M M_1} - 2 K_2 C_M \right)$  is plotted as a function of  $C_M^2$  and from the limiting slope at  $C_M^2 = 0$ ,  $K_3$  is calculated. The operation is repeated till the entire data fit into a straight line and the slope gives the constant for the formation of the highest polymer existing in the equilibrium mixture.

An analysis of the data, by this procedure, shows that at  $\mu = 0.20$  only dimers and trimers are formed;  $K_2 = 0.09$  &  $K_3 = 0.22$ . At  $\mu = 0.10$  up to hexamers are formed and  $K_2 = 0.45$ ,  $K_3 = 0.05$ ,  $K_4 = 0.10$ ,  $K_5 = 0.14$  and  $K_6 = 0.36$ . The data indicates the existence of heptamers also, but, in view of the scatter in experimental points at high concentration, no attempt was made to evaluate  $K_7$ . Similarly, at  $\mu = 0.05$  hexamers (or heptamers) seem to be the highest polymer formed and  $K_2 = 4.0$ ,  $K_3 = 0.10$ ,  $K_4 = 2.6$ ,  $K_5 = 1.2$  and  $K_6 = 0.48$ . These constants are calculated on the concentration scale of grams per litre.

It should be noted that in the procedure used for the analysis of the data any uncertainty involved in the estimation of a  $K$  value is carried over to the evaluation of the successive  $K$ 's. The  $K$  values are subject to considerable uncertainty because of this and also due to the scatter in the experimental  $\bar{M}_w$  data. Still, the  $K$  values at  $\mu = 0.05$  appear



to be almost ten times greater than the corresponding values at  $\mu=0.10$ . At a given protein concentration there is a greater preponderance of the polymers at  $\mu=0.05$  than at  $\mu=0.10$  and consequently the measured  $\overline{M}_w$  values are higher. But both at  $\mu=0.05$  and at  $\mu=0.10$ , the highest type of polymer formed is a hexamer or a heptamer.

The sedimentation velocity behaviour of such systems is of considerable interest. Earlier theories<sup>15,16</sup> predict a single boundary if the association-dissociation reaction is infinitely rapid but more than one boundary if it is slow. However, Gilbert<sup>17</sup> has shown theoretically that even for infinitely rapid reaction more than one boundary may be expected under certain conditions. According to Gilbert, if diffusion is neglected and if the intermediate polymers are absent, one boundary results if only dimers are formed and two boundaries will occur for higher polymers. This theory explains the sedimentation velocity data of Massey *et al.*<sup>11</sup> on  $\alpha$ -chymotrypsin.

It is seen that at  $\mu=0.20$ , only dimers and trimers of  $\alpha$ -chymotrypsin are formed. Sedimentation velocity experiments give only one boundary (Fig. 4C) and on the basis of Gilbert's theory, two peaks should be expected. Gilbert had assumed for the sake of mathematical simplicity that only the monomer and the highest polymer are present and the intermediates are absent. Rao and Kegeles<sup>8</sup> showed, however, by a straight forward extension of Gilbert's theory that a mixture of monomers, dimers and trimers should give a single boundary. They, thus, found Gilbert's theory adequate to explain the experimental data.

At  $\mu=0.10$ , where upto hexamers are formed, the sedimentation velocity pattern consists of a single boundary and at  $\mu=0.05$  where also upto hexamers are formed the pattern consists of two peaks (Figs. 4 A & B). One should expect two boundaries at  $\mu=0.10$  also. Recently, Gilbert<sup>18</sup> has postulated from theoretical calculations that the shape of the boundary is affected by the greater stability of one polymer species to make it dominant in the equilibrium mixture. An idea of the relative abundance of the various species may be obtained from the calculated equilibrium constants. Such calculations may reveal the reasons for obtaining one boundary at  $\mu=0.10$  and two at  $\mu=0.05$ . These computations are in progress and the results will be published elsewhere.

## Summary

The application of the Archibald method of molecular weight determination to chemically reacting systems has been described. A study of the polymerization of  $\alpha$ -chymotrypsin in phosphate buffers of ionic strength ( $\mu$ ) 0.05, 0.10 and 0.20 has been made. The extent of polymerization decreases with increase in ionic strength. In sedimentation velocity experiments a single boundary is obtained at  $\mu=0.10$  and 0.20, and two peaks at  $\mu=0.05$ .

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### Discussion

- Q. Could the method be applied to the study of protein-protein interactions?
- A. Yes—by applying methods analogous to those illustrated in the case of chymotrypsin association-dissociation.
- Q. What is the influence of temperature on the sedimentation velocity pattern?
- A. The effect of temperature has not been studied. A large effect may not be expected as the temperature coefficient of sedimentation is low.
- Q. Is the method applicable to low molecular weight proteins?
- A. Yes, provided the molecular weight is above the prescribed minimum.



# FRACTIONATION ISOLATION AND ELECTROPHORETIC CHARACTERIZATION OF RED GRAM GLOBULINS

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Only a limited amount of exact information is available concerning the isolation and separation of plant proteins<sup>1-4</sup>. The present investigations were carried out with a view to developing methods for the isolation of homogeneous protein fractions from red gram (*Cajanus cajan*) meal and to study their properties by moving boundary electrophoresis.

## Experimental Materials and Methods

**Material:** Defatted red gram meal used in these studies was prepared according to the method described earlier<sup>5</sup> by passing the meal through 80 mesh B.S. seive and defatting with petroleum ether.

**Differential Solubility:** The meal was extracted successively with water, 10 per cent sodium chloride and 0.25 M sodium hydroxide. It was also extracted with 75 per cent alcohol separately. The nitrogen distribution in the extracts was determined by the procedure described by Nath and Giri<sup>4</sup>, using the micro-Kjeldahl method (Table I). Non-protein nitrogen was determined after precipitation of proteins with 0.8 M trichloroacetic acid.

TABLE I. Successive extraction of red gram meal with different extractants

Extractant	Total nitrogen dispersed* %
Water ... ..	14.30 (albumin, globulin and non-protein nitrogen)
10% Sodium chloride ... ..	74.36 (globulin nitrogen)
0.25 M Sodium hydroxide ... ..	4.79 (glutelin nitrogen)
75% Alcohol ... ..	2.28 (prolamine nitrogen and non-protein nitrogen)
0.8 M Trichloroacetic acid ... ..	2.09 (non-protein nitrogen)

\* Mean of three observations

**Dialysis:** Sodium chloride extract was dialysed against water and the precipitated globulins were redissolved in phosphate buffer of pH 7.0 and ionic strength 0.1, and analysed electrophoretically. The supernatant was also analysed under the same conditions.

**Ammonium sulfate fractionation:** This was carried out according to the procedure of Jones *et al.*<sup>3</sup>. Fractions, obtained at 20, 40 and 60 per cent saturation, were analysed electrophoretically (Table II).

**Dilution and fractional precipitation:** 10 per cent sodium chloride extract of the meal buffered to pH 7.0 with phosphate buffer, was diluted ten times with water when a white precipitate was obtained. The precipitate was redissolved in 10 per cent sodium chloride



TABLE II. *Electrophoretic analysis of red gram globulin fractions obtained by ammonium sulfate fractionation of 10% NaCl extract*  
(pH 8.0, ionic strength 0.1, temp. 25°C.)

Fraction (obtained with ammonium sulphate saturation at)		$-u \times 10^5 \text{ cm}^2 \text{ volt}^{-1} \text{ sec}^{-1}$					
		I		II		III	
		<i>a</i>	<i>d</i>	<i>a</i>	<i>d</i>	<i>a</i>	<i>d</i>
20%	...	8.25	7.56	11.25	10.62	...	...
40%	...	8.09	7.21	11.68	11.05	13.25	12.78
60%	...	...	...	10.22	9.78	13.52	15.13
		Relative area %					
		I		II		III	
		<i>a</i>	<i>d</i>	<i>a</i>	<i>d</i>	<i>a</i>	<i>d</i>
20%	...	...	...	...	...	...	...
40%	...	80.11	83.65	19.89	16.35	...	...
60%	...	21.25	20.05	35.75	32.50	43.0	47.45
		...	...	39.25	32.40	60.75	57.60

TABLE III. *Electrophoretic analysis of red gram globulin fractions obtained by dilution and fractional precipitation with ammonium sulphate*  
(pH 8.0, ionic strength 0.1, temp. 25°C.)

Fraction		$-u \times 10^5 \text{ cm}^2 \text{ volt}^{-1} \text{ sec}^{-1}$					
		I		II		III	
		<i>a</i>	<i>d</i>	<i>a</i>	<i>d</i>	<i>a</i>	<i>d</i>
I	...	7.89	7.42	...	...	...	...
II	...	8.05	7.59	11.20	11.34	14.52	13.91
III	...	...	...	12.32	11.12	14.69	14.21
		Relative area %					
		I		II		III	
		<i>a</i>	<i>d</i>	<i>a</i>	<i>d</i>	<i>a</i>	<i>d</i>
I	...	100.00	100.00	...	...	...	...
II	...	11.55	13.68	72.23	78.52	16.22	7.80
III	...	...	...	30.21	22.13	69.79	77.87

and reprecipitated by dilution (Fr. I, Table III). The supernatant was fractionated with ammonium sulfate and two fractions were obtained at 40 per cent (Fr. II) and 60 per cent (Fr. III) saturation. All the fractions were thoroughly dialysed against distilled water in cold and dried over phosphorus pentoxide *in vacuo* at 4°C. The details of this fractionation scheme have been described previously<sup>4</sup>.



All the fractions were analysed electrophoretically by the moving boundary method. Fraction I, which was the major component, was analysed at various pH values to determine its homogeneity and isoelectric point. Other fractions were analysed at pH 8.0 only.

## Results and Discussion

The results of the experiment on the successive extraction of proteins in water, 10 per cent sodium chloride solution and 0.25 M sodium hydroxide solution have been presented in Table I. From the results (Table I), the absence of prolamine nitrogen and low content of glutelin nitrogen can be observed. Though 10 per cent sodium chloride solution solubilizes the major portion of the globulins, water was also found to extract globulins to some extent. This was apparent when electrophoretic analysis revealed three components in either limb at pH 7.0 (Fig. 1). The electrophoretic patterns of sodium chloride (Fig. 1a) and water extract (Fig. 1b) were similar except for the higher concentrations of the components in the former.

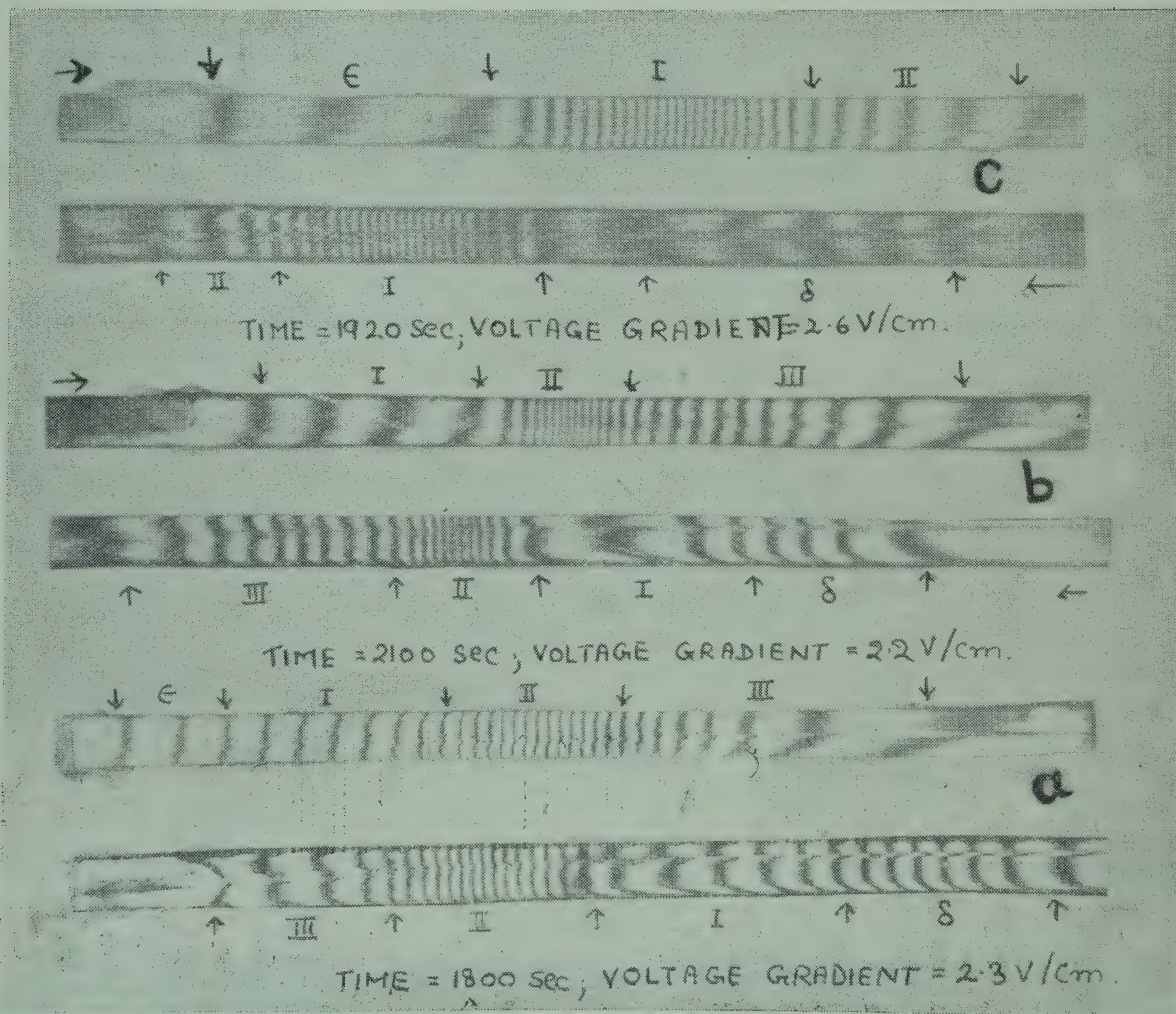


FIG. 1. Electrophoretic analysis of (a) sodium chloride extract (b) water extract and (c) fraction obtained by dialysing sodium chloride extract against water.



TABLE IV. Electrophoretic mobilities of fraction I at different pH values, ionic strength 0.1, temp. 25°C

Expt. No.	pH*	$-u \times 10^5 \text{ cm}^2 \text{ volt}^{-1} \text{ sec}^{-1}$	
		<i>a</i>	<i>d</i>
1	3.0	+ 3.08	+ 2.97
2	4.0	+ 1.18	+ 1.02
3	4.5	+ 0.47	+ 0.48
4	5.0	- 1.08	- 0.91
5	6.0	- 3.53	- 3.25
6	7.0	- 5.92	- 4.65
7	8.0	- 7.89	- 7.42
8	9.1	- 9.55	- 9.32
9	10.0	-10.48	-10.69
10	11.0	-11.22	-10.98
11	12.0	-13.12	-12.56

\* Composition of buffers: Expt. No. 1, 0.1M sodium acetate -0.097M HCl; Expt. No. 2, 0.1M sodium acetate -0.4M acetic acid; Expt. No. 3, 0.1M sodium acetate -0.23M acetic acid; Expt. No. 4, 0.1M sodium acetate -0.05M acetic acid; and Expts. No. 5-11, same as given by Nath and Giri<sup>4</sup>.

*a*: ascending pattern.

*d*: descending pattern.

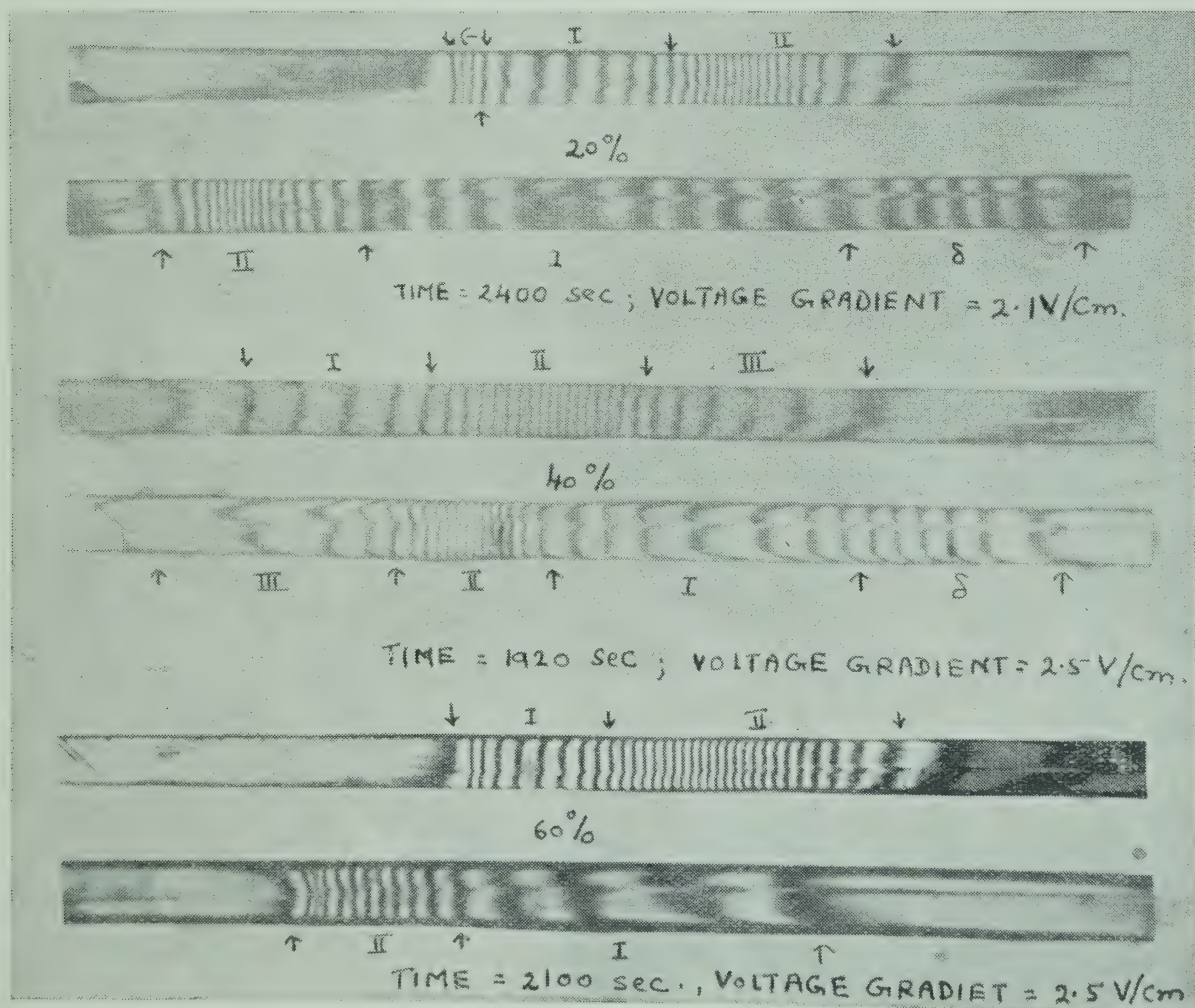


FIG. 2. Electrophoretic analysis of fractions obtained at 20, 40 and 60% ammonium sulfate saturation. (pH 8.0; ionic strength 0.1; temp. 25°C)



Dialysis of sodium chloride extract against water yielded a fairly homogeneous fraction, slightly contaminated with another component (Fig. 1c).

The fractions obtained at 20, 40, and 60 per cent saturation with ammonium sulfate were electrophoretically heterogeneous and consisted of two, three and two components respectively (Fig. 2, Table II).

Of all the methods used, the method of dilution and fractional precipitation was found to be most satisfactory for isolation of red gram globulin fractions and resulted in the preparation of a major component in an electrophoretically homogeneous state (Table III).

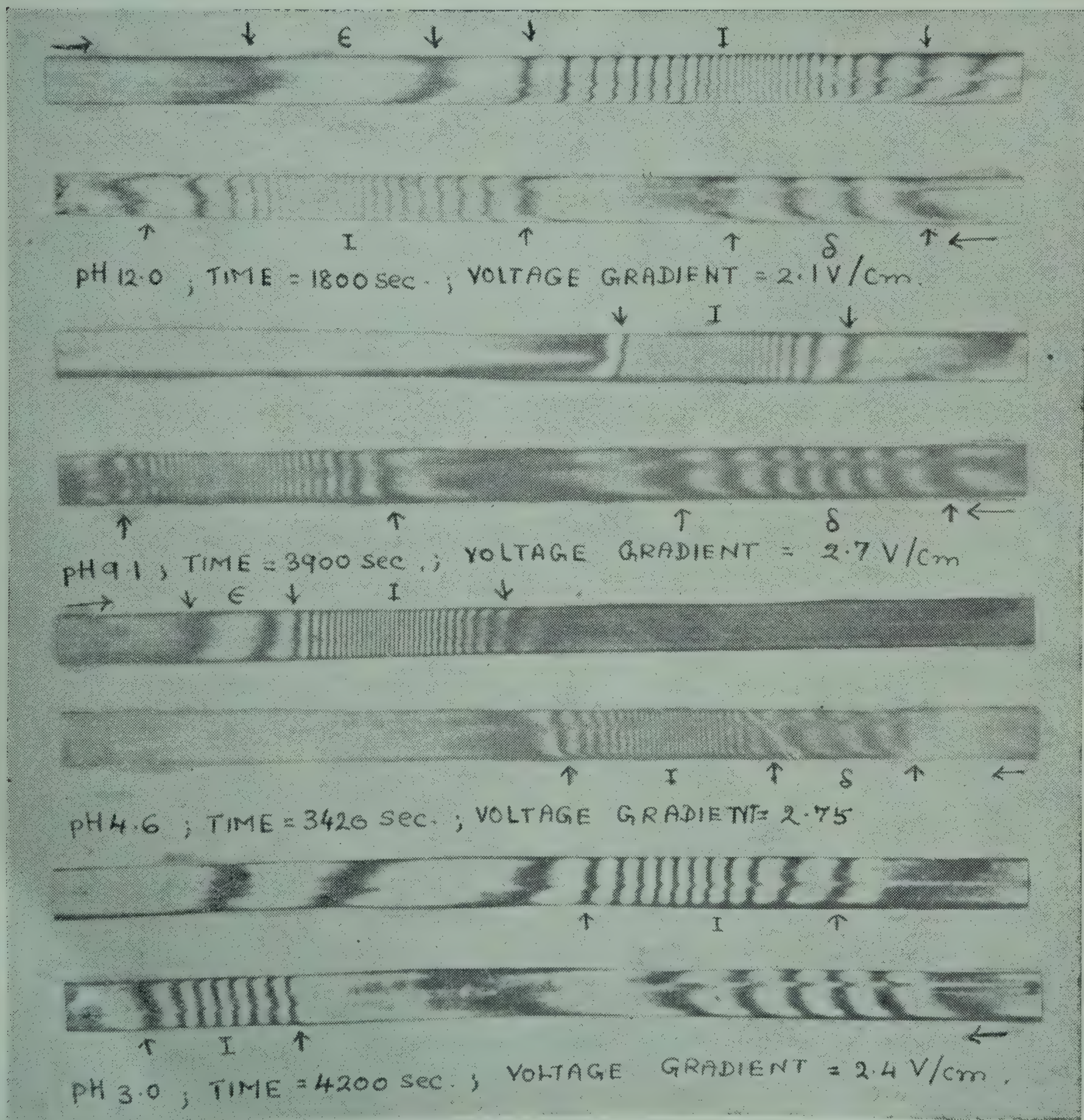
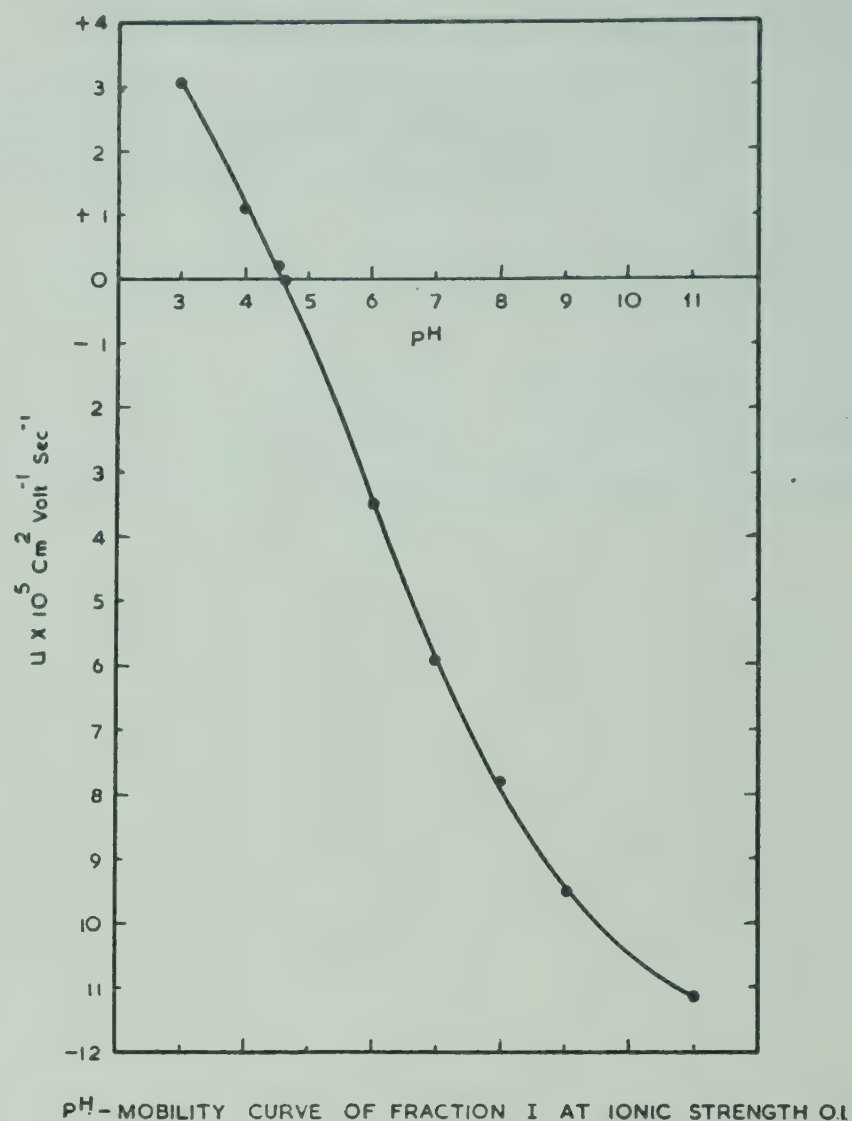


FIG. 3. Electrophoretic analysis of fraction I at different pH values and voltage gradients. (ionic strength 0.1)



It can be observed from the data in Table III and Fig. 3 that while fraction I shows a single boundary, the other two fractions are contaminated. Fraction II is contaminated with both the fractions I and III, while fraction III is a mixture of fractions II and III.

Fraction I contained nearly 75 per cent of the protein and was analysed electrophoretically in detail over the pH range 3.0 to 12.0 (Table IV). The buffers used were the same as given by Nath and Giri<sup>4</sup>. The results are presented in Table IV and Fig. 3. It can be seen that fraction I behaves as a homogeneous protein throughout the pH range tested. The isoelectric point of this fraction as determined from the mobility curve is at pH 4.65. (Graph 1). Nath and Giri<sup>6</sup>, following the same fractionation scheme, obtained a fraction from sesame proteins which was homogeneous over the pH range 4.5 to 12.0 but gave two and three components at pH 4.0 and 4.25 respectively.



GRAPH 1

## Summary

The fractionation of red gram proteins has been carried out by (1) extraction with different solvents, (2) dialysis of sodium chloride extract against water, (3) fractional precipitation with ammonium sulfate and (4) dilution and fractional precipitation with ammonium sulfate. The various fractions were tested for their homogeneity electrophoretically. The dilution and fractional precipitation method gave most satisfactory results, and one major globulin fraction which was electrophoretically homogeneous between pH 3.0-12.0 with isoelectric point of 4.65, was obtained. Among the other methods used, only dialysis of sodium chloride extract against water yielded a fairly homogeneous fraction.

## Acknowledgment

The authors' thanks are due to the Council of Scientific and Industrial Research, New Delhi, for financial assistance.

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*Discussion*

Q. Do the various electrophoretic fractions differ in amino acid make-up?

A. The amino acid composition of these fractions is yet to be studied.

Q. Have electrophoretic separations been carried out at different ionic concentrations?

A. No.

Q. What is the quantitative yield of the homogeneous fraction?

A. 75 per cent of the original protein.



# HETEROGENEITY OF BUFFALO HAEMOGLOBIN

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Haemoglobin heterogeneity has assumed great importance since the discovery of an abnormal haemoglobin in sickle-cell anaemia by Pauling and co-workers<sup>1</sup>. The important contributions of Ingram<sup>2,3,4</sup> on the structural manifestation of abnormality in human haemoglobins have added impetus to research in this field. Multiple haemoglobins have been reported in many animals like rabbit<sup>5</sup>, cow<sup>5</sup>, sheep<sup>6</sup>, horse<sup>7</sup>, monkey<sup>8</sup>, rat<sup>5,9</sup> and buffalo<sup>9</sup>. In most of these cases, only one or two criteria were employed to establish heterogeneity.

This paper presents the results of studies on the heterogeneity of the buffalo haemoglobin using four different criteria, namely, alkali denaturation, salting-out with ammonium-sulphate, electrophoresis and ion-exchange chromatography. It has been possible to show that the blood of the normal buffalo contains three haemoglobins.

## Experimental Materials and Methods

*Haemolysate:* Blood was collected from the jugular vein of healthy adult buffaloes in oxalate solutions and the red cells were separated by centrifugation. The cells were washed repeatedly with 0.9 per cent sodium chloride and centrifuged. The plasma-free red cells were haemolysed with an equal volume of water. The insoluble material was removed from the haemolysate by centrifugation in the cold for 20 minutes at 10,000 rpm. The supernatant was saturated with carbon monoxide. The carboxyhaemoglobin, thus obtained, was employed in all the studies.

*Alkali denaturation:* Alkali denaturation studies were carried out according to the procedure of Haurowitz *et al.*<sup>5</sup>. The kinetics of denaturation were followed spectrophotometrically at 540 m $\mu$ .

*Salting-out studies:* Salting-out studies were made using 4 M ammonium sulphate according to the method of Allison and Tombs<sup>10</sup>.

The haemolysate was diluted 1:20 with 1 M phosphate buffer of pH 6.8. To 1 ml of buffered haemolysate, in a series of tubes, volumes of water varying from 8 ml to 1 ml were added. 4 M ammonium sulphate was introduced to make the volume in each tube exactly 10 ml. The tubes were closed tightly by means of rubber bungs and the contents mixed by inversion. They were equilibrated at  $24^{\circ}\text{C} \pm 1^{\circ}\text{C}$  for 24 hours. The contents in each tube were filtered and their optical density measured at 540 m $\mu$ .

*Agar electrophoresis:* The procedure was similar to that of Giri<sup>11</sup>. Electrophoresis was carried out at pH 8.6 in veronal-acetate buffer of ionic strength 0.05.

*Ion-exchange chromatography:* Amberlite IRC-50 was pre-treated according to the method of Hirs *et al.*<sup>12</sup>. The chromatographic procedure was similar to that of Huisman and Prins<sup>13</sup> using 0.1 M sodium citrate-citric acid buffer of pH 6.0. The dimensions of the column were  $40 \times 2.8$  cm and the resin bed occupied a height of 25 cm. 100 mg of haemolysate in 0.1 M citrate-citric acid buffer of pH 6.0 was loaded on the column. All chromatographic operations were performed at  $0-4^{\circ}\text{C}$ .



*Optical density:* All optical density measurements were made in a Beckman Model DU Spectrophotometer.

## Results

Fig. 1 shows the plot of  $\log n$ , ( $n$  is the percentage of native haemoglobin) against time. The plot can be fitted into two straight lines intersecting at the twelfth minute. This indicates that the haemolysate contains two haemoglobins. By extrapolating the portion CB to cut the ordinate, the slow denaturing protein was found to be about 98 per cent of the total haemoglobins.

Solubility studies revealed greater heterogeneity. The plot of the optical density of the filtrate against salt concentration (Fig. 2) showed three components AB, BC and CD.

Two components are seen in electropherograms (Fig. 3) as reported by Giri and Pillai<sup>9</sup>. The relative concentrations of the fast moving component and the slow moving component were 63 and 37 per cent respectively. These did not correspond to the quantitative values obtained in this study by alkali denaturation. The salting-out studies

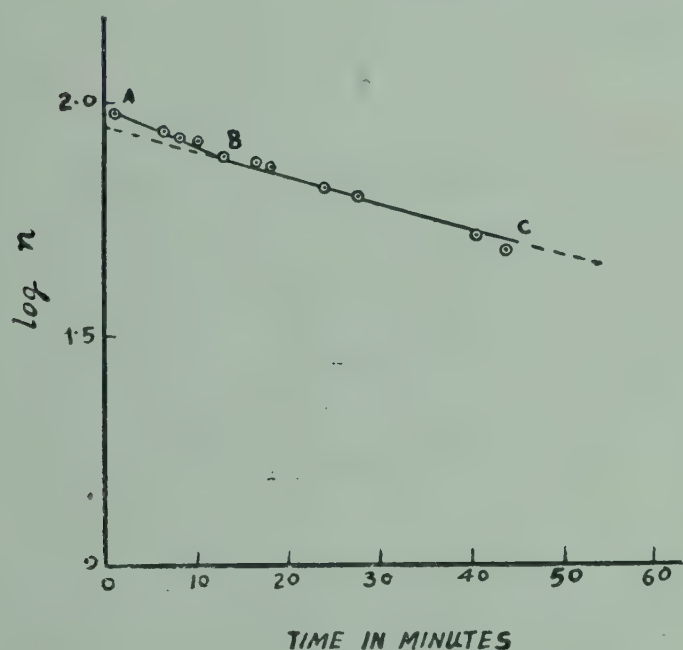


FIG. 1. Denaturation plot of buffalo haemolysate in 0.05N NaOH at 26°C.

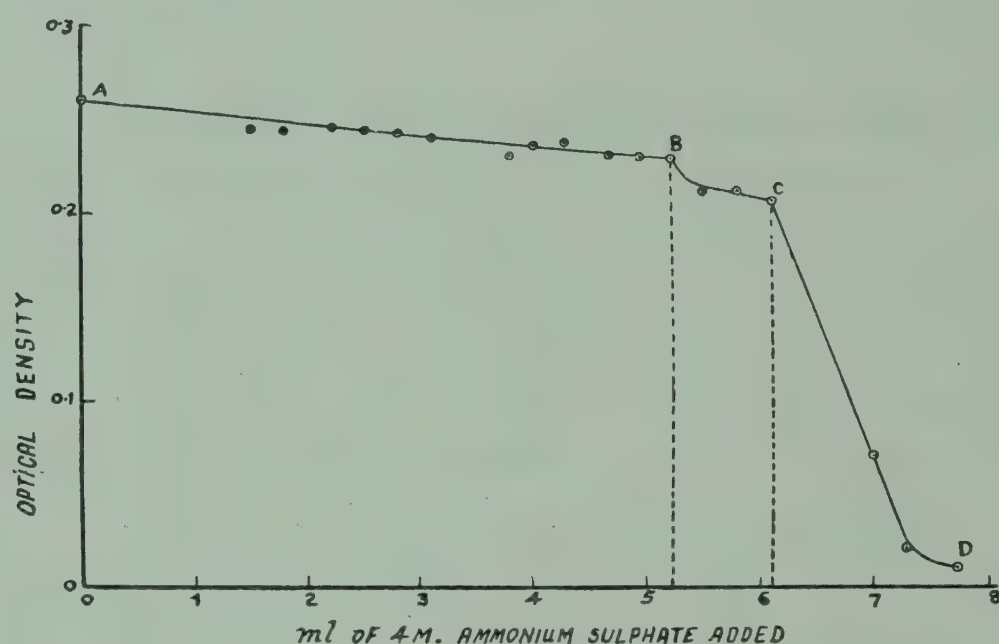


FIG. 2. Solubility curve of buffalo blood haemolysate

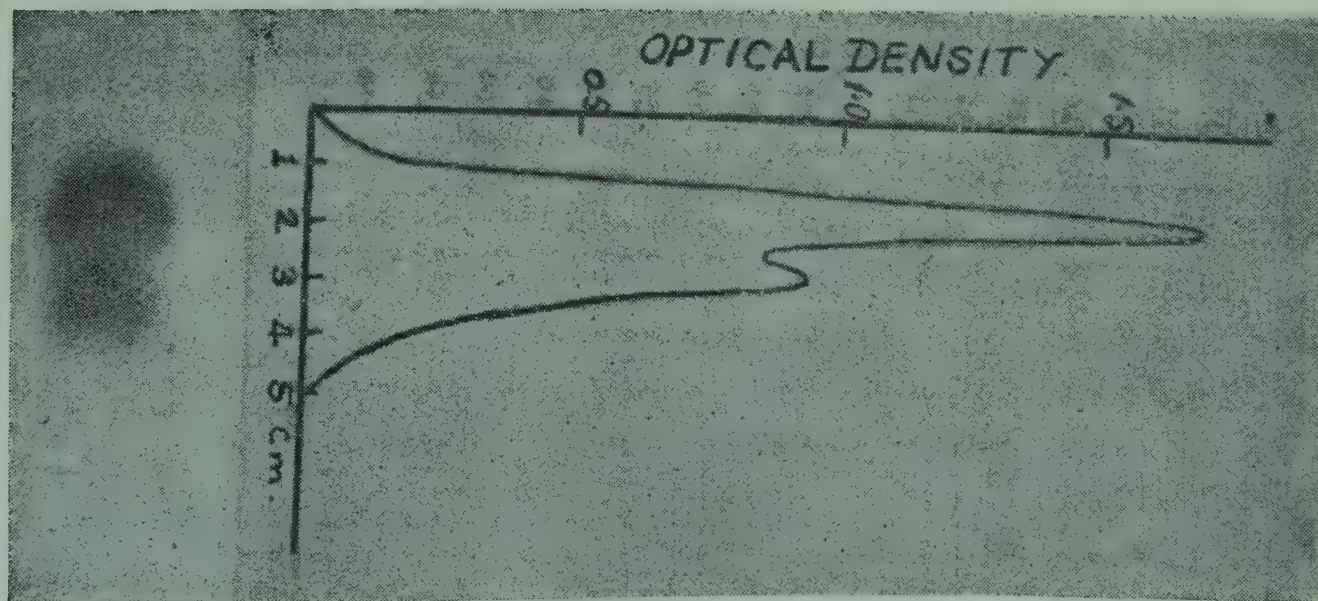


FIG. 3. Agar electrophoretic pattern of buffalo haemolysate Veronal-acetate buffer, pH 8.6, ionic strength 0.1; 7 volts per cm; 5 mA; Time of run 6 hr.



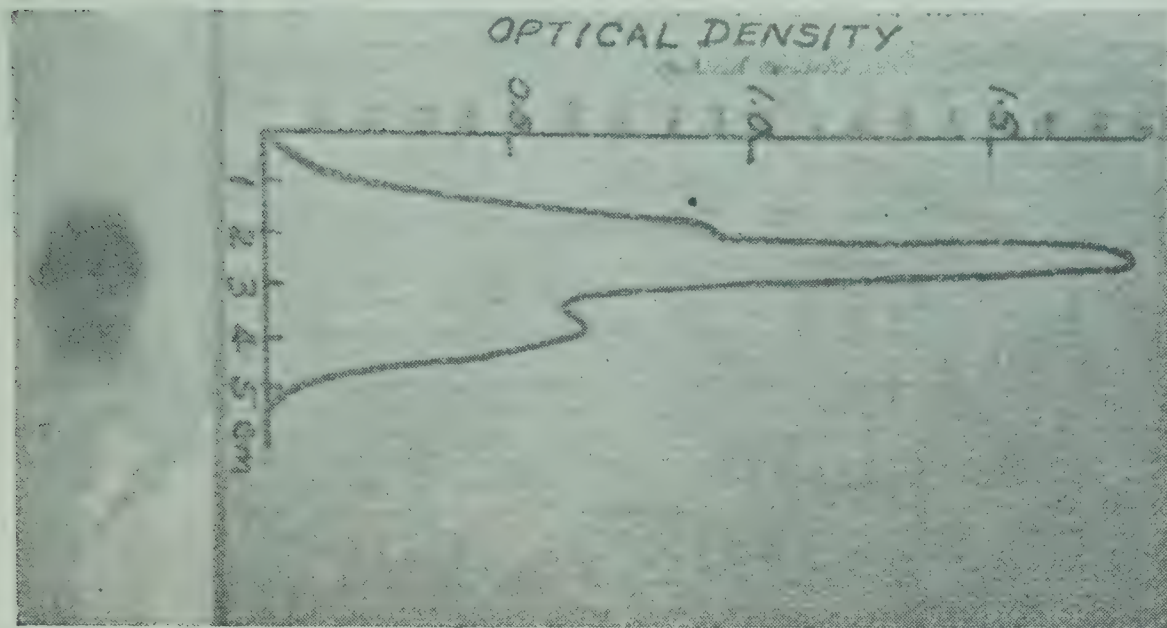


FIG. 4. Agar electrophoretic pattern of buffalo haemolysate showing the third component as a tailing. Conditions of experiment as in Fig. 3. Time of run 10 hr.

indicated the possibility of a third component being present. Agar electrophoresis for longer periods (10 hr) showed a third component (Fig. 4) moving faster than the other two, reminiscent of the patterns of Kunkel and Wallenius<sup>14</sup>. The densitometric pattern reveals this component as a shoulder. By measuring the areas corresponding to the

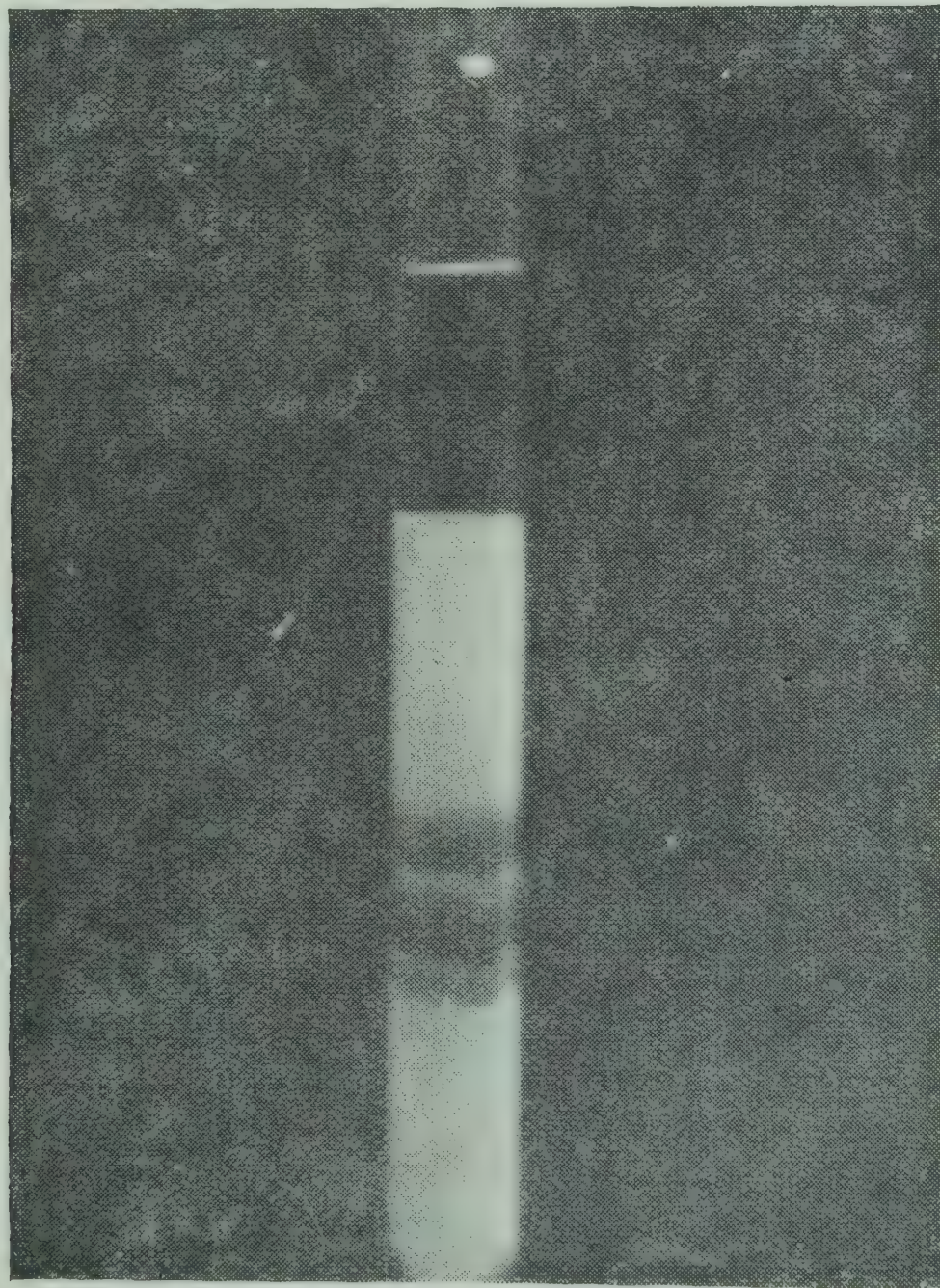


FIG. 5. Column chromatogram of buffalo haemolysate showing the three haemoglobins. 0.1 M sodium citrate-citric acid buffer, pH. 6.0.



three zones, it was found that the fast moving band comprised 12 per cent, the major band 63 per cent and the slow band the remainder of the haemoglobins.

Final confirmation of the heterogeneity was obtained from separation of the components on columns of IRC-50. The two components which separate in agar electrophoresis during short intervals (6 hr.—Fig. 3), separated on IRC-50 columns when 100 ml of the buffer were run through the column. On passing an additional 100 ml. of the buffer, a small band which had the highest rate of migration separated out. All the three components could be separated out as discrete zones by continuing the chromatography. Fig. 5 shows a typical chromatogram.

## Discussion

The heterogeneity of the buffalo haemoglobin has been clearly established. Alkali denaturation showed the presence of two components. After 12 minutes the haemoglobin behaved as a single protein and was quite resistant to alkali. In the resistance of the second component to alkali it resembles cow haemoglobin, but the haemoglobin denaturing in 12 min. resembles rabbit haemoglobin<sup>5</sup>. Apart from the data on heterogeneity, this study demonstrated the presence of two haemoglobins, varying widely in their resistance to alkali, though they are synthesised in the same animal. The variation in the linkage between the prosthetic group and the globin accounts for the difference in the rates of denaturation<sup>5</sup> as the prosthetic groups are identical and globins of different species denature at the same rate. This may be true of haemoglobins synthesised in the same animal. Human foetal haemoglobin is more resistant to alkali than normal adult haemoglobin and the difference is attributed to different sites of synthesis. It is probable that the sites of synthesis may not be the same for all the three haemoglobins of the buffalo also.

Solubility studies furnished evidence for the presence of a third component. The relative percentages of the three components are not in agreement with those observed by alkali denaturation studies. These criteria are, however, not reliable for quantitative evaluation, but only useful in checking homogeneity<sup>15</sup>. Zone electrophoresis, regarded as an accepted method of haemoglobin analysis<sup>15</sup>, revealed three components and actual estimation of the relative concentrations was made by this method. The third component, obtained as a tailing similar to the patterns of Kunkel and Wallenius<sup>14</sup>, is found for the first time in these electrophoretic studies. It is to be expected that the same order of migration should be observed on columns of a cation-exchanger. The order of separation was the same both in electrophoresis and chromatography. These bands are not artefacts as shown by the fact that blood samples from twelve different normal buffaloes behaved identically. Furthermore, on elution from the columns and subsequent characterisation, the haemoglobins differ in acidic amino acid content and thiol group content and show variations in the N-terminal amino acids<sup>16</sup>.

## Summary

The haemolysate of the normal adult buffalo has been examined for heterogeneity using alkali denaturation, salting out, zone electrophoresis and ion exchange chromatography. Alkali denaturation indicated two haemoglobins, while salting out and zone



electrophoresis on agar gel indicated three haemoglobins. Column chromatography using IRC-50 confirmed the presence of three haemoglobins. This study establishes the presence of three haemoglobins in the buffalo blood. The third haemoglobin has been reported for the first time.

### Acknowledgment

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### Discussion

- Q. Could the heterogeneity observed be ascribed to partial denaturation?
- A. Haemoglobin is known to be resistant to mild procedures of denaturation. Moreover we have used a number of criteria to establish the distinctive character of the components.
- Q. How does the elution pattern from the ion-exchange resin correspond with the chromatographic pattern?
- A. Such comparative studies have not been carried out.
- Q. Could the electrophoretic components arise through dissociation?
- A. Dissociation starts only below pH 5.8. The electrophoresis was carried out in the alkaline range.



# ISOLATION OF AN ELECTROPHORETICALLY HOMOGENEOUS $\alpha_1$ -MUCOPROTEIN FROM SHEEP PLASMA

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Despite extensive investigations on the chemical and biological properties<sup>1-8</sup> of human plasma  $\alpha_1$ -acid glycoprotein (also called 'Orosomucoid' or  $\alpha_1$ -mucoprotein), little is known about similar proteins of other animal species. Investigations of Weimer and Winzler<sup>9</sup> showed the possibility of wide chemical variations in plasma  $\alpha_1$ -mucoproteins of different animals. Information on the isolation and characterisation of sheep plasma  $\alpha_1$ -mucoprotein is not available.

The present communication deals with the isolation of an electrophoretically homogeneous  $\alpha_1$ -mucoprotein from sheep plasma by a combination of the method of Weimer *et al*<sup>3</sup>. and preparative agar electrophoresis<sup>10</sup>.

## Experimental

Agar (B.D.H.), veronal sodium (B.D.H.) and sodium acetate trihydrate (A.R.) were used without further purification. Ammonium sulphate (Riedel-de-Hean A.G.) was recrystallized before use.

Nitrogen estimations were carried out by the micro-Kjeldahl procedure.

*Isolation of sheep plasma  $\alpha_1$ -mucoprotein:* The method described by Weimer *et al*<sup>3</sup>. was followed for the isolation of sheep plasma  $\alpha_1$ -mucoprotein. Plasma globulins were first precipitated at 50 per cent saturation of ammonium sulphate. The bulk of the albumin was precipitated by adjusting the pH of the filtrate to 4.5 and 3.7.  $\alpha_1$ -mucoprotein was finally precipitated from the filtrate by full saturation with ammonium sulphate. The mucoprotein precipitate was thoroughly dialysed and concentrate  $\alpha$  over phosphorous pentoxide *in vacuo* at 5°C. The isolated dry substance was dissolved in water (5°C) to give a 2 per cent solution, pH adjusted to 3.7 and the protein reprecipitated by full saturation with ammonium sulphate. The precipitate obtained was dissolved in the minimum amount of water and dialysed thoroughly against water in the cold. The dialysate was dried as before. In spite of re-fractionation, the final product obtained was admixed with as much as 10 per cent albumin. In order to separate albumin from  $\alpha_1$ -mucoprotein, the preparative agar electrophoresis procedure was applied to the mucoprotein fraction isolated as described above.

*Preparative agar electrophoresis for the isolation of  $\alpha_1$ -mucoprotein:* The procedure described by Das and Giri<sup>10</sup> was followed. 200 mg mucoprotein, isolated as above, was dissolved in 2 ml veronal buffer, pH 8.6 and ionic strength ( $\mu$ ) 0.05. 200 ml agar-buffer mixture was prepared by mixing equal volumes of hot 1 per cent autoclaved agar solution with veronal buffer, pH 8.6 and  $\mu=0.1$ . About 20 ml hot 1 per cent agar solution was layered on a perspex electrophoretic trough which served as the preparative cell (Fig. 1). After 15 minutes, when the agar layer had set into a solid foundation, at either ends of the perspex trough Whatman No. 3 filter papers, cut to suitable size, were introduced.



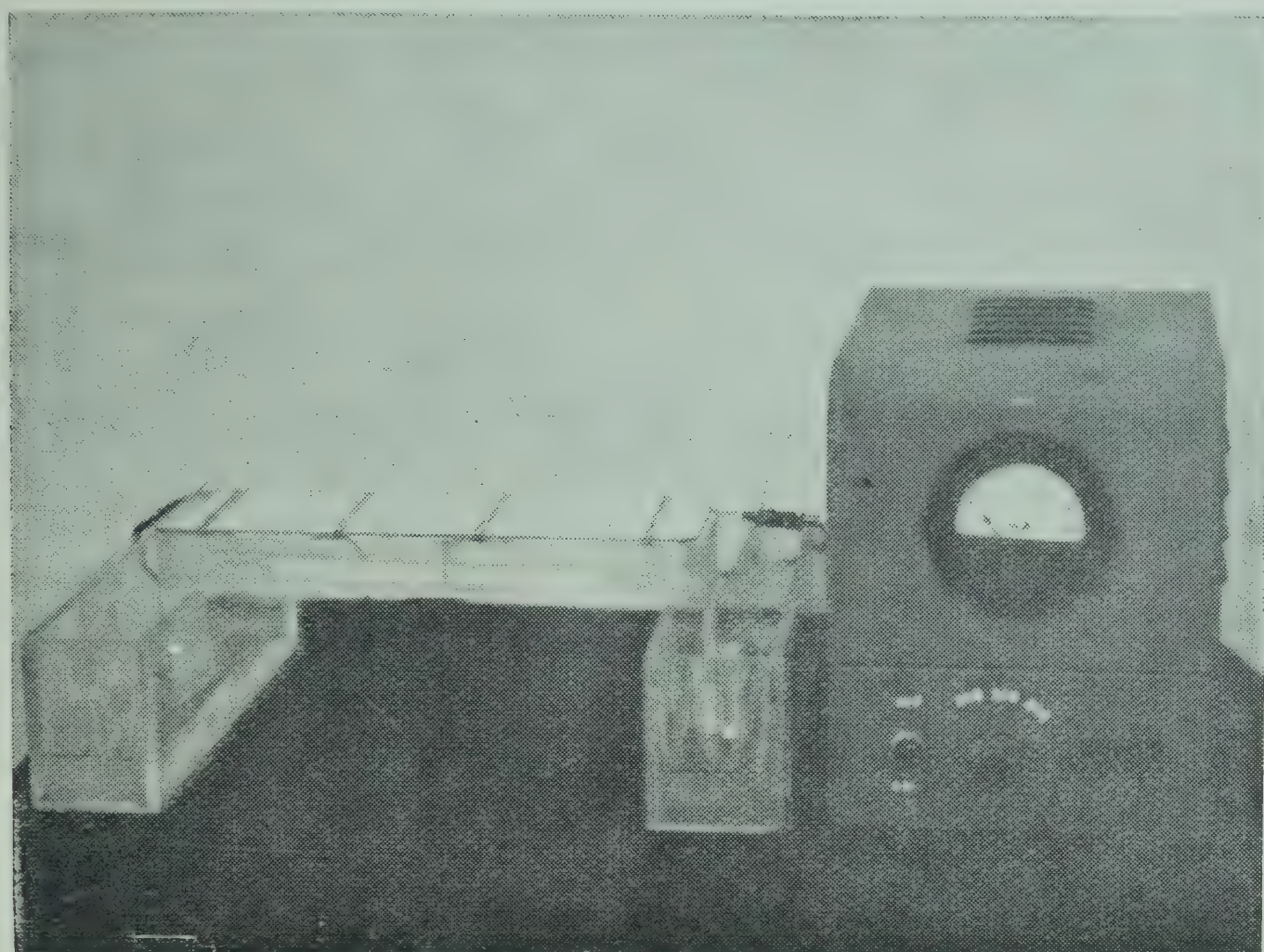


FIG. 1. Preparative Agar Electrophoresis unit

The trough was now filled with 200 ml agar-buffer mixture. A small rectangular perspex piece was introduced at the centre of the agar-buffer mixture and held in position by means of a clamp till the agar set into a firm gel (about 45 minutes). The perspex trough with agar gel, filter papers and central perspex piece was now kept on the electrode vessels containing veronal buffer (pH 8.6,  $\mu$  0.05) so that the filter papers dipped into the buffer (Fig. 1). The central perspex piece was carefully removed from the gel and in the resulting slit, mucoprotein solution was poured carefully by means of a capillary pipette. The perspex trough was covered with a lid and electrophoresis was conducted at 300 volts, 8 milliamps, for 18 to 20 hours at room temperature (22°-25°C). After electrophoresis, the area occupied by albumin and  $\alpha_1$ -mucoprotein was identified by a test print on Whatman No. 1 filter paper as described by Das and Giri<sup>10</sup>. The agar gel corresponding to each zone was cut out and carefully transferred into separate tubes, and frozen at -20°C for about 3 hours. The tubes were then allowed to thaw at room temperature, the thawed solution was filtered over a sintered glass funnel and thoroughly dialysed in the cold (5°C) against large volumes of water for about 24 hours. The dialysates were made to definite volumes and nitrogen was estimated on suitable aliquots. The total nitrogen recovered from each fraction was calculated and the per cent protein recovered was expressed on nitrogen basis. The remaining amounts of dialysates were dried over phosphorous pentoxide *in vacuo* at 5°C. The dried fractions thus obtained were more homogeneous electrophoretically.

The preparative agar electrophoresis unit, used in the present investigation, is shown in Figure 1.



## Results and Discussion

The agar electrophoretic pattern of sheep plasma  $\alpha_1$ -mucoprotein obtained by the procedure of Weimer *et al.*<sup>3</sup> is shown in Figure 2a. Figure 2b represents the same protein after refractionation showing the relative increase in the amount of  $\alpha_1$ -mucoprotein.

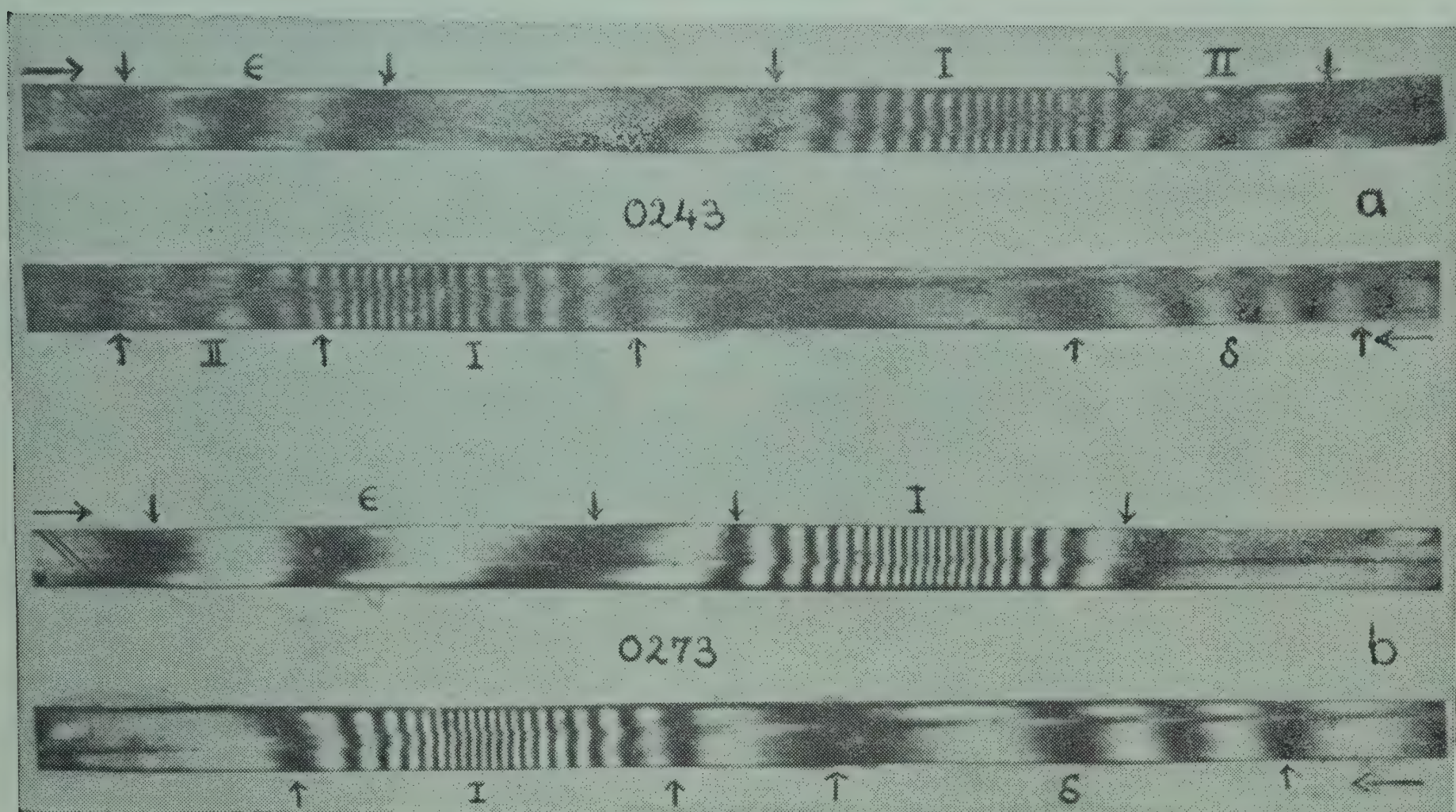


FIG. 3. Electrophoretic patterns of sheep plasma  $\alpha_1$ -mucoprotein isolated by (a) Winzler's method, (b) preparative agar electrophoresis pH 8.6, time 2700 sec. and voltage gradient 4.2 v/cm.

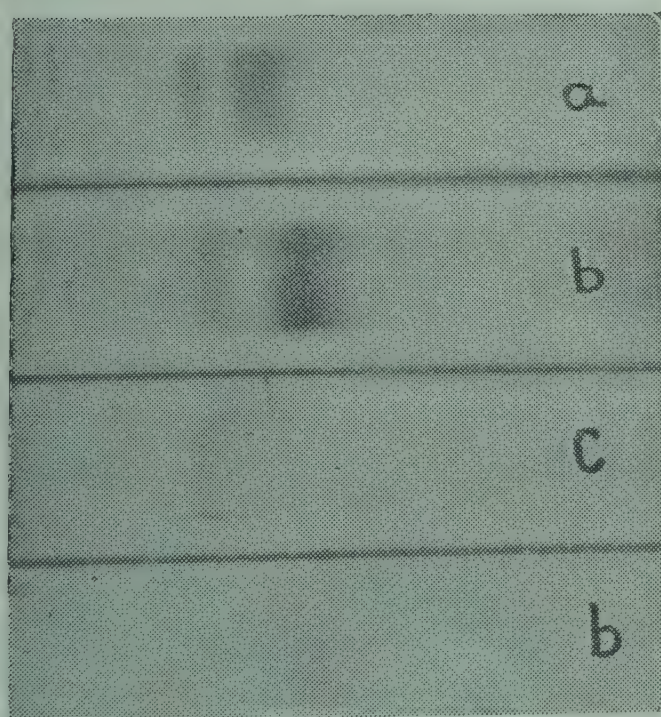


FIG. 2. Agar electrophoretic patterns of mucoprotein: (a) Isolated by Winzler's procedure, (b) Refractionated. After preparative agar electrophoresis, (c) Albumin, (d)  $\alpha_1$ -mucoprotein.

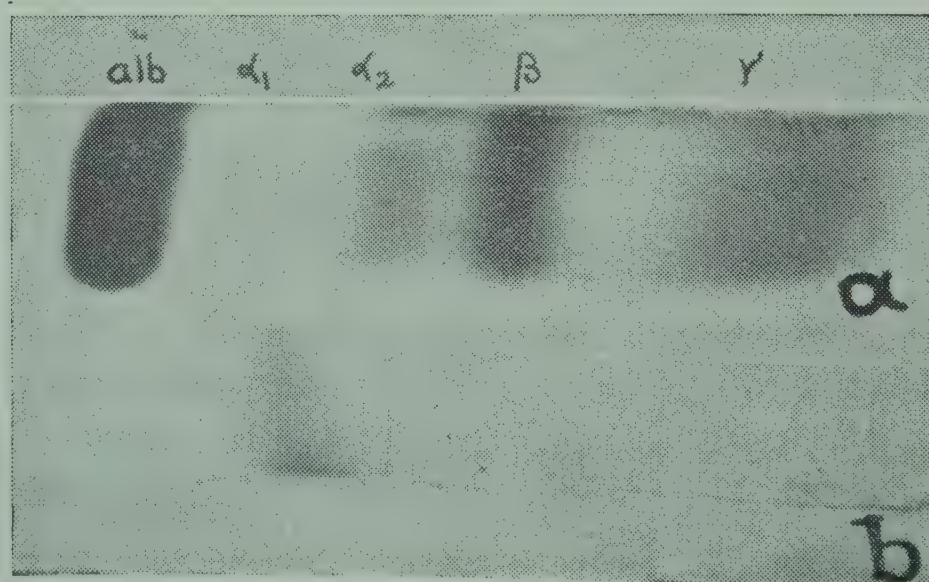


FIG. 4. Agar electrophoretic patterns of : (a) sheep plasma, (b) isolated  $\alpha_1$ -mucoprotein. alb—Albumin,  $\alpha_1$ ,  $\alpha_2$ ,  $\beta$ - and  $\gamma$  — globulins.



The fractions of  $\alpha_1$ -mucoprotein and albumin, isolated after the application of preparative agar electrophoresis, can be made out from Figures 2c and 2d, respectively.

Typical interferometric patterns of sheep plasma  $\alpha_1$ -mucoprotein before and after isolation by preparative agar electrophoresis, obtained on Kern's moving boundary micro-electrophoresis apparatus are shown in Figure 3. It is evident from the patterns that the mucoprotein isolated by preparative agar electrophoresis is more homogeneous.

Results of some qualitative tests made on isolated sheep plasma  $\alpha_1$ -mucoprotein are presented in Table I. In view of its positive biuret and Molisch tests, non-coagulability on boiling at 100°C, solubility in 1.8 M perchloric acid and 10 per cent trichloroacetic acid and precipitability on full saturation with ammonium sulphate and with 5 per cent phosphotungstic acid in 2 N hydrochloric acid, the protein is to be classified as a mucoprotein. Since it has the same electrophoretic mobility as plasma  $\alpha_1$ -globulin, it is designated sheep plasma  $\alpha_1$ -mucoprotein (Fig. 4).

TABLE I. *Colour reactions and effect of protein precipitants on isolated sheep plasma  $\alpha_1$ -mucoprotein*

Test	Results
Biuret	Positive
Molisch	Positive
Boiling at 100°C	No coagulation
<b>Effect of precipitants:</b>	
<i>Effective</i>	<i>Not effective</i>
(i) 5% phosphotungstic acid in 2 N HCl.	(i) 1.8 M Perchloric acid
(ii) Full saturation with ammonium sulphate	(ii) 10% Trichloroacetic acid

In Table II, the per cent recovery of proteins after preparative agar electrophoresis is given. Three different samples of mucoprotein, isolated from different batches of blood, showed about 85 per cent recovery.

TABLE II. *Recovery of sheep plasma  $\alpha_1$ -mucoprotein after preparative agar electrophoresis*

Sl. No.	Batch	1*	2*	3*	Average
1.	Amount of mucoprotein taken (mg.)	150	165	180	165
2.	Nitrogen content of the sample (%)	13.0	13.5	12.8	13.1
3.	Corresponding amount of nitrogen in mucoprotein taken (mg.)	19.5	22.27	23.04	21.6
4.	Nitrogen recovered after preparative agar electrophoresis (mg.)				
	In albumin zone	1.71	2.20	1.97	1.96
	In $\alpha_1$ -globulin zone	15.52	18.00	16.45	16.86
5.	Per cent of nitrogen recovered in albumin zone	84.85	83.00	90.04	85.96
	in $\alpha_1$ -globulin zone	88.54	91.72	75.61	85.29

\* Per cent of albumin present in mucoproteins of batch 1, 2 and 3 were 10.1, 11.9 and 9.5 respectively. These determinations were made by electrophoretic analysis on agar.



Thus, starting with one litre of sheep plasma it has been possible to obtain 200-300 mg. of mucoprotein by the procedure of Weimer *et al.*<sup>3</sup> and by preparative agar electrophoresis as much as 150-200 mg/litre plasma, of an electrophoretically homogeneous  $\alpha_1$ -mucoprotein has been obtained.

## Summary

Sheep plasma  $\alpha_1$ -mucoprotein has been isolated in an electrophoretically homogeneous state by the combination of Winzler's procedure and preparative agar electrophoresis.

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## Discussion

- Q. What was the hexosamine content of the mucoprotein? Has homogeneity been related to the ratio of hexosamine to protein nitrogen at different stages of purification?
- A. The hexosamine content of the mucoprotein was 8-10 per cent. The correlative studies have not been carried out.



# USE OF ION-EXCHANGERS IN THE SEPARATION OF PROTEINS OF COBRA VENOM

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It is well known that snake venoms are mainly composed of proteins some of which are enzymic in character. Some of the more important enzymes present in cobra venom are: protease, lecithinase, nucleotidase, phosphodiesterase cholinesterase and L-amino acid oxidase<sup>1</sup>. In addition, cobra venom contains neurotoxins and an anticoagulant whose nature or mode of action is not known. It was the aim of this work to separate the constituents of the venom of the Indian Cobra (*Naja naja*) and to study the properties of the individual components.

Master and Rao<sup>2</sup> have separated the cobra venom on a micro-scale using the starch gel electrophoresis method of Smithies<sup>3</sup> and Poulik<sup>4</sup>. Under these conditions cobra venom separated into 10 components. A few of the enzymes could be identified on the starch gel itself by using chromogenic substrates and histochemical procedures developed in this laboratory<sup>2,5</sup>. Thus phosphomonoesterase, phosphodiesterase, cholinesterase and lecithinase could be identified. Other components such as protease, anticoagulant principle, L-amino acid oxidase and neurotoxin were identified by eluting the starch gel strips after electrophoresis and testing the eluates. It was found that the toxic component did not coincide with any of the enzymes reported above.

The conventional methods of separation of proteins from complex mixtures, such as fractionation with ammonium sulphate or precipitation with alcohol or acetone in the cold, did not give homogeneous fractions as tested by toxicity, gel diffusion and electrophoresis. The use of ion-exchange resins or cellulose ion-exchangers was tried in the hope of better results. When this work was in progress some reports of the use of ion-exchangers for such fractionations were published. Doery<sup>6</sup> has used IRC-50 for fractionation of the Australian tiger-snake venom and cellulose ion-exchangers have been used by other workers<sup>7,8,9</sup>. The ion-exchangers IRC-50, diethylaminoethyl (DEAE) cellulose and carboxymethyl (CM) cellulose ion-exchangers were used for fractionation in the present work.

## Materials and Methods

IRC-50 was obtained from the British Drug House.

DEAE (Diethylaminoethyl) cellulose was obtained as a gift from Serva-Entwicklungslabor, Heidelberg.

CM (Carboxymethyl) cellulose was prepared from cellulose powder by the method of Peterson and Sobers<sup>10</sup>.

## Enzyme activities

*Esterase*: Phenylbutyrate substrate was prepared from phenol and butyryl chloride by the method of Sumner *et al.*<sup>11</sup>. Esterase activity was measured colorimetrically at pH 9.0 in tris buffer using phenol reagent as described by the same authors with very slight modification.



*Phosphodiesterase* activity was measured by the method of Sinsheimer and Koerner<sup>12</sup> using calcium bis-*p*-nitrophenyl phosphate as the substrate at pH 8.9 in tris buffer. The liberated *p*-nitrophenol was measured colorimetrically at 420 m $\mu$ .

*L-amino acid oxidase* activity was qualitatively detected by a paper chromatographic method developed in this laboratory. The acid formed by the action of venoms on L-histidine at pH 8.0 in phosphate buffers was separated from the amino acid by circular paper chromatography using 77 per cent ethyl alcohol as the solvent and detected by spraying with diazotized sulphanilic acid.

*Protease* was detected by placing 0.05 ml of fractions or their dilutions on the surface of an exposed but undeveloped photographic strip and incubating in a humid incubator. Suitable controls were always included. The highest dilution of the venom fraction producing a clear zone was taken as the end point for comparing activities.

*Lecithinase* activity was detected by incubating the venom fractions with 0.1 per cent lecithin in saline for one hour and adding washed sheep cells as an indicator for observing the haemolysis.

*Anticoagulant activity* was determined by measuring prothrombin time by Quick's one stage method. The prothrombin time of recalcified normal human serum varied between 3 to 8 min. The highest dilution increasing this time to 1 hour was taken for comparing anticoagulant activity of venom fractions.

*Toxicity* of the fractions was determined by injecting 0.5 ml of dilutions intravenously into mice weighing 20 g.

*Non-dialyzable protein* was estimated by dialyzing the fractions thoroughly and estimating protein according to the method of Lowry<sup>13</sup> using phenol reagent containing copper sulphate and compared with the colour values obtained with dialyzed cobra venom solutions containing known amount of the venom.

### *Chromatographic procedures*

Among the ion-exchange resins tested the cation-exchanger IRC-50 was found to fractionate better. It was used in the H—form after activation with 1.5 N HCl. This was poured into a column 35×2.5 cm. and washed with water and finally with 0.2 M phosphate buffer of pH 6.8. 300 mg of cobra venom dissolved in 6 ml of phosphate buffer was added at the top and the column was developed by using phosphate buffers of molarity increasing to 0.4 M. The out flow was at the rate of 10 ml/hr and 4 ml fractions were collected. Optical density of the fractions was measured at 280 m $\mu$  in a Beckman spectrophotometer and the values were plotted. The number of peaks indicated the separation of the venom into fractions. The solutions under individual peaks were pooled and labelled. The individual pooled fractions were concentrated either by pervaporation through cellophane tubes or by freeze-drying in an Edwards centrifugal freeze drier. The fractions were then tested for enzymic and other activities.

2 g. of DEAE cellulose was allowed to swell in water and regenerated with 0.5 N NaOH. It was then washed free from the alkali by centrifugation and finally washed with 0.02 M phosphate buffer at pH 7.0. After removing the finer particles, which did not easily sediment, it was poured into the chromatographic column 1 cm. in diameter where it occupied a length of 12.7 cm. Two ml of 10 per cent cobra venom solution was added and the chromatogram was developed using gradient elution technique with 0.02 M



phosphate buffer containing increasing molar concentrations of NaCl. The rate of flow was 8 ml/hr and fifty 1.0 ml fractions were collected. The rest of the procedure was the same as for IRC-50.

The procedure used with CM cellulose was exactly the same as that with DEAE cellulose.

## Results

The cation-exchange resin IRC-50 showed the presence of 10 components (I-1 to I-10) in cobra venom as indicated in Table I. It was however, found that L-amino acid oxidase could not be detected in any of the fractions by the paper chromatographic technique. Fraction I-3 had the maximum amount of esterase activity. The same fraction also had a trace of phosphodiesterase activity, most of which seemed to have been lost. Toxicity was highest in fractions I-5 and I-7 and these two components also had the anticoagulant principle.

TABLE I

Fraction No.	Solids (mg/cc)	Esterase (O.D./mg.)	Toxicity activity (mg.)	Anticoagulant activity (mg.)	Lecithinase
I 1	0.20	0.82	...	...	...
I 2	0.02	14.12	...	...	...
I 3	0.22	0.70	...	...	...
I 4	0.06	0.293	...	...	...
I 5	0.31	0.027	0.015	0.0031	++
I 6	0.22	0.240	...	...	...
I 7	0.56	0.223	0.0056	0.00056	...
I 8	0.24	0.070	...	...	...
I 9	0.02	9.160	...	...	...
Venom	...	0.7696	0.0083	0.001	4+

In view of the destruction or inability to elute the enzymes, L-amino acid oxidase and phosphodiesterase, under the conditions of the experiment, the ion-exchange celluloses were tried next. Gradient elution of cobra venom from DEAE cellulose showed the presence of 12 components D-1 to D-12 as shown in Table II. Fraction 3 had the highest total solids.

The enzyme and other activities of the DEAE fractions are given in Table II. The values are calculated per mg of non-dialyzable proteins in the fractions and compared with the values for the whole venom. The values are not expressed in absolute units but give only a comparative idea. It is seen that esterase activity is highest in fractions D-6 and D-8. The phosphodiesterase activity was, however, found in fractions D-3, 5, 8 and 10. The highest activity being in fraction D-3. Fraction D-3 had also the highest toxicity and fraction D-6 had very little, the other fractions being practically non-toxic. L-amino acid oxidase activity was present in fractions D-3 and D-9; it was more in the former. The anticoagulant activity was present in fraction D-3 only.



TABLE II

Fraction No.	Solids (mg./ml).	Esterase (O.D./mg.)	Phosphatase (O.D./mg.)	Toxicity (mg.)	Anticoagulant activity (mg.)	Oxidase	Lecithinase	
D 1	...	0.17	1.50	0.025	...	...	1+	...
D 2	...	0.36	0.72	0.012	...	...	1+	...
D 3	...	1.50	0.022	0.1203	0.000015	0.0018	4+	2+
D 4	...	0.76	0.719	0.0031	0.00076	0.09	1+	4+
D 5	...	1.00	0.620	0.0362	0.01	0.15	...	1+
D 6	...	0.47	4.09	0.010	0.0047	0.059	1+	1+
D 7	...	0.34	1.20	0.012	...	...	1+	...
D 8	...	0.11	3.10	0.040	...	...	1+	...
D 9	...	0.14	0.50	...	...	...	3+	...
D 10	...	0.655	0.64	0.04	...	...	...	...
D 11	...	0.163	1.02	0.150	...	...	...	...
D 12	...	0.15	0.47	0.0140	...	...	...	...
Venom	...		0.7696	0.1427	0.0083	0.001	4+	4+

Since fraction D-3 had the highest protein concentration and toxicity, and was also rich in the enzymes phosphodiesterase and L-amino acid oxidase, it was analyzed by gel diffusion and was found to contain 8 components. It was therefore decided to concentrate and refractionate this fraction on the cation-exchanger CM cellulose.

Fraction D-3 was freeze-dried in Edward's centrifugal freeze drier and made into a 10 per cent solution and fractionated on CM cellulose. CM cellulose resolved it into 6 fractions C-1 to C-6. The results are given in Table III.

It is seen that CM cellulose has resolved esterase into 3 fractions in fraction C-2 and C-4. The toxic component was concentrated in fraction C-4. The L-amino acid oxidase activity, phosphodiesterase activity and anticoagulant activity were still found together in fraction 3.

TABLE III

Fraction No.	Solids (mg./ml).	Oxidase	Esterase (O.D./mg.)	Phosphatase (O.D./mg.)	Toxicity (mg.)	Anticoagulant activity (mg.)	Lecithinase
C 1        ...	0.25	...	...	0.0352	...	...	...
C 2        ...	0.158	4+	1.35	0.892	0.039	0.000015	4+
C 3        ...	2.18	1+	0.047	0.109	0.072	0.00210	1+
C 4        ...	0.345	...	0.573	0.185	0.0172	0.00423	...
C 5        ...	0.423	...	0.0021	0.105	0.0211	0.020	...
C 6        ...	2.000	...	...	0.0132	...	...	...
Orig. Fr. D 3	...	4+	0.022	0.1203	0.000015	0.0018	4+



## Discussion

The fractionation of complex mixtures of proteins as in snake venom is a very difficult procedure. The starch-gel electrophoretic method is applicable only for the separation of micro-quantities of the venom. The ion-exchange resin IRC-50 gave rise to fairly well defined peaks during elution. However, since some of the enzymes like L-amino acid oxidase and phosphodiesterase could not be recovered with IRC-50, the method had to be given up in preference to the cellulose ion-exchanger.

Use of the cellulose ion-exchangers is preferable since they have high capacity, the eluting conditions are very mild and there are less chances of inactivation of the enzymes. It however suffers from one disadvantage; the eluates are dilute and have to be concentrated. Freeze-drying procedure was found to be very convenient to get concentrated solutions. A combination of anionic DEAE cellulose and the cationic CM cellulose has given better results.

The above work is only of a preliminary nature. Further work is now in progress to prepare larger quantities of the purer fractions of cobra venom and study the nature and mode of action of the biochemically or pharmacologically more important components.

## Summary

1. Cobra venom has been fractionated on a preparative scale using IRC-50 and DEAE and CM cellulose ion-exchangers.

2. The fractions have been tested for their enzyme activities such as esterase, phosphodiesterase, protease, L-amino acid oxidase, lecithinase, anticoagulant activity and also for toxicity.

3. DEAE cellulose in combination with CM cellulose has been found to give a good separation of the constituents of cobra venom following a gradient elution procedure.

## Acknowledgment

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*Discussion*

- Q. What was the basic underlying idea in examining all fractions for toxicity and enzyme activity? Are enzymes implicated in the toxicity?
- A. Quite often enzyme activity coincided with toxic activity.
- Q. Which are the enzymes known to be toxic?
- A. Proteinases and diesterase.
- Q. Did the protein elution pattern (on the ion-exchangers) follow the enzyme elution pattern?
- A. There was no such correlation.
- Q. Was there any hydrolysis on IRC-50 columns?
- A. There was no evidence of hydrolysis. All these experiments were done at low temperature.



# STUDY OF PROTEIN-PROTEIN INTERACTIONS IN SERUM UNDER THE INFLUENCE OF HEAT

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The antitoxic activity of immune sera deteriorates at the rate of about 10 per cent per year even when stored in the cold. An excess unitage has always to be added to cover deterioration during the validity period declared on the label. The rate of deterioration is accelerated at higher temperatures. Storage at 37°C or at higher temperatures for a few months causes gelification. The reasons for such deterioration of potency or for gelification are not known, though denaturation of the proteins has been considered the main cause of these changes. The term denaturation cannot be defined exactly and depends on the property or activity of the protein being measured. The object of this work was to investigate the changes brought about in serum proteins under the influence of heat between 50°C and 70°C for half an hour.

## Experimental Materials and Methods

*Normal horse sera and anti-diphtheria sera* from hyperimmunized horses were obtained from the Department of Immunology, Haffkine Institute. Diphtheria toxin and international standard diphtheria antitoxins were also obtained from the same source.

*Paper electrophoresis* was carried out on Whatman No. 3 MM paper in barbiturate buffer of pH 8.6, ionic strength 0.075 and containing 10 per cent glycerine. After electrophoresis for about 18 hours the papers were dried at 105°C and stained with bromophenol blue.

*Immuno-electrophoresis* was carried out according to the method of Grabar<sup>1</sup>.

*Antitoxic content* of anti-diphtheria sera was measured *in vitro* by flocculation procedure and *in vivo* by the rabbit skin test as described in the Indian Pharmacopoeia. International standard diphtheria antitoxin was always used as reference standard.

## Results

I. *Effect of heat on normal horse serum*: Normal horse serum was taken from a sample of pooled sera of a few horses which were kept without immunization. The serum was distributed in 10 ml quantities into test-tubes. They were diluted with equal volumes of water. The first tube was kept as control and the other five were heated at temperatures of 50°, 55°, 60°, 65° and 70°C respectively for half an hour. The tubes were immediately cooled under the tap. In the tube heated at 65°C and 70°C there was increase in viscosity but no gel formation. Heating of undiluted sera at 65°C and 70°C was found to give rise to gel formation. 0.01 ml of sample from each of the six tubes was subjected to paper electrophoresis. Figure 1 is a photograph of the stained strips. Table I gives the quantitative data on the protein composition of the sera subjected to heat as above and Figure 2 is a photograph of immuno-electrophoresis.



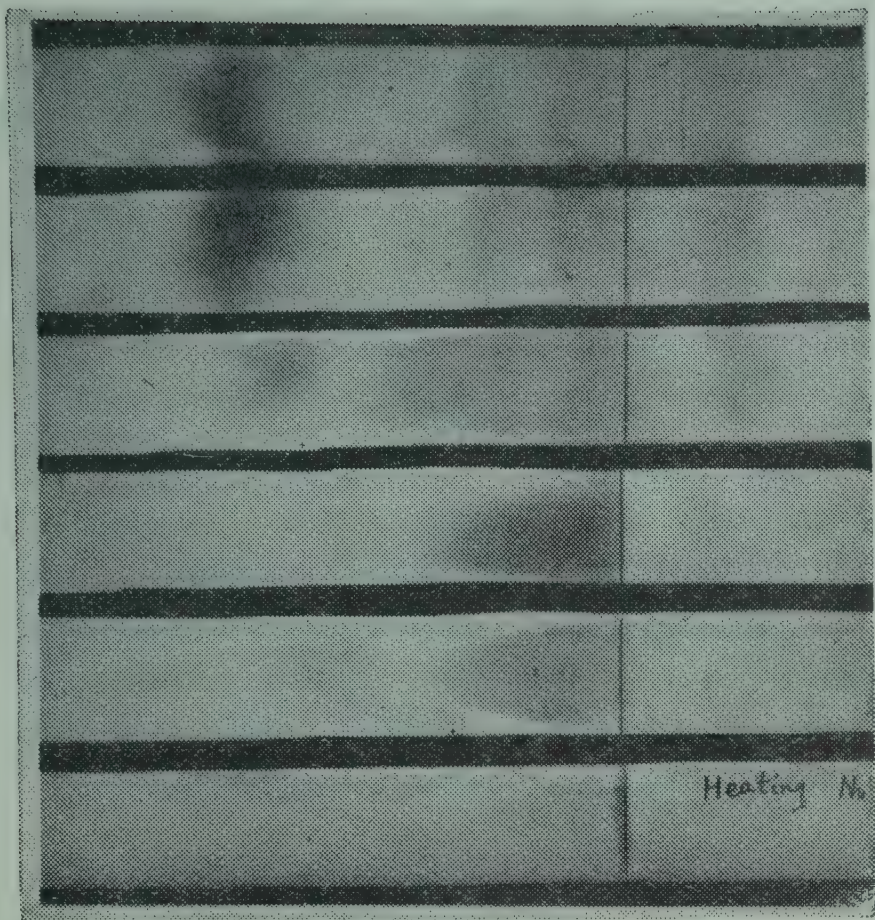


FIG. 1. Paper electrophoresis of normal horse serum heated for 30 min. at 50° to 70°C

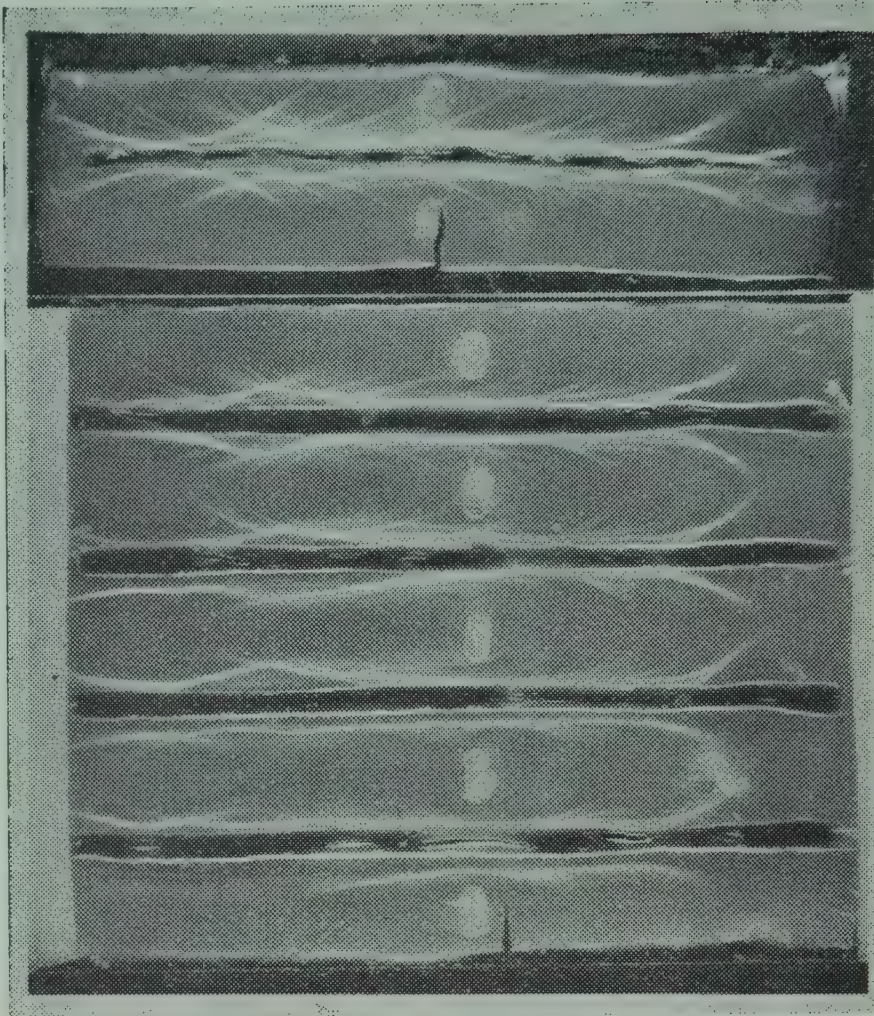


FIG. 2. Immuno-electrophoresis of normal horse serum heated for 30 min. at 50° to 70°C

TABLE I. *Electrophoretic analysis of heated horse sera*

Protein fraction		Control	50°C	55°C	60°C	65°C
Albumin	...	44.1%	32.7	16.8	3.6	0
$\alpha_1$	...	4.3%	5.0	63.9	75.6	90.9
$\alpha_2$	...	9.2%	12.8			
$\beta$	...	23.5%	26.0			
$\gamma$	...	19.0%	23.5	19.3	20.9 (includes precipitate at point of application)	9.1

It is seen from the electrophoretic pattern in Figure 1 that there was hardly any change in serum proteins kept at 50°C for half an hour. The changes take place markedly at 55°C and above. The first change to be noticed was the decrease in the quantity of albumin and rise in that of  $\alpha_2$ -globulin. There was also a slight decrease in  $\gamma$ -globulin. At 60°C, there was very little albumin and  $\gamma$ -globulin, mostly  $\alpha$  and  $\beta$  globulins which also did not show any separation between them. These changes were more pronounced at 65°C and 70°C.

The results of immuno-electrophoresis (Figure 2) support the results of electrophoresis. The former brought out more clearly the formation of protein-protein complexes under



the influence of heat. The number of arcs progressively diminished. At 65°C, for example, there was one precipitin line right from the albumin region and reaching out almost to the  $\gamma$ -globulin region. There was also residual albumin and  $\gamma$ -globulin which have the same mobility as unheated albumin, indicating that heating does not change the electrophoretic mobility but only brings about protein-protein combination.

II. *Effect of heat on horse serum albumin:* Serum albumin was prepared from normal horse serum by repeatedly precipitating with ammonium sulphate between 50 per cent and 75 per cent. The albumin was found to be homogeneous by electrophoresis. Aliquots of a 2 per cent solution of this albumin were heated at 55°, 60°, 65° and 70°C for 30 minutes. The solutions heated at 65° and 70°C became slightly cloudy but there was no gel formation. The solutions were then subjected to electrophoresis. Figure 3 is a photograph of the electrophoresis strips.

It is seen from Figure 3 that there is no change in electrophoretic mobility under the influence of heat. The formation of a slightly cloudy solution and increase in viscosity at 65° and 70°C perhaps indicates polymerization.

III. *Effect of heating diphtheria antitoxic sera on its antitoxic titre:* It is possible to estimate the antitoxic  $\gamma$ -globulin by the use of diphtheria antitoxic sera for studying the effects of heat and get an idea of the changes in the protein.

Diphtheria antitoxic sera were diluted with an equal volume of water and aliquots heated as before at temperatures between 50° and 70°C. Figure 4 is a photograph of the electrophoretic patterns of the solution. It will be noticed that the pattern resembles closely that for normal horse serum in Figure 1. The heated samples were tested for their antitoxic titre using both the *in vitro* flocculation method as well as the *in vivo* rabbit skin test. Results are given in Table II.

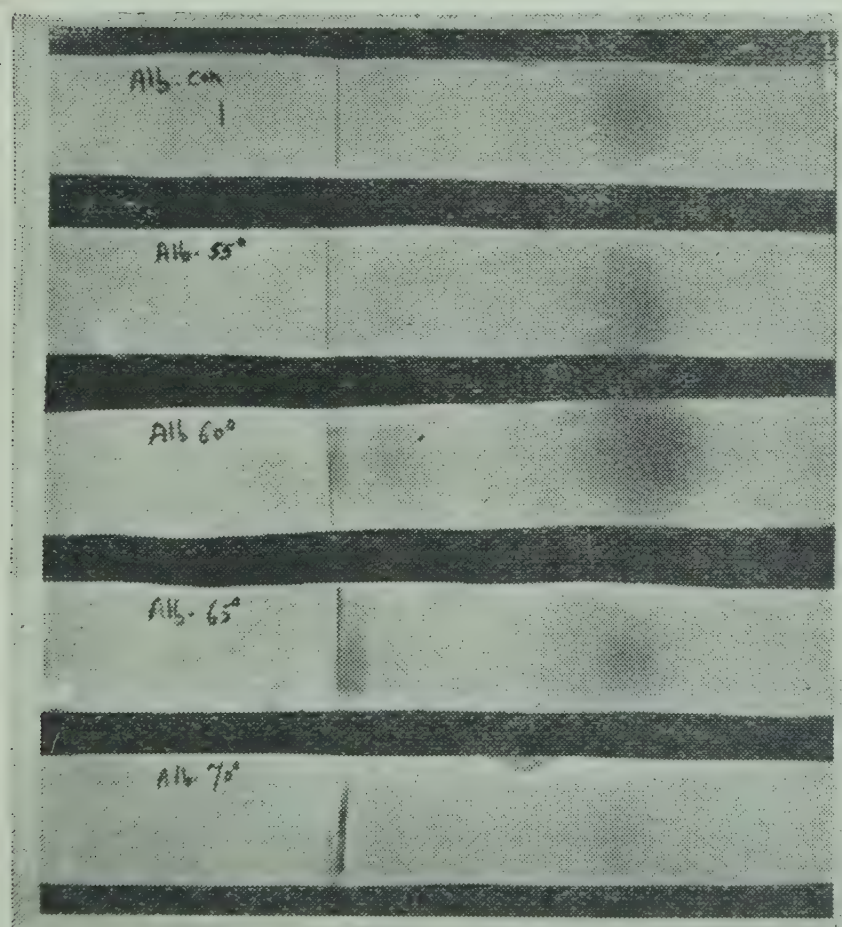


FIG. 3. Paper electrophoretic pattern of serum albumin heated for 30 min. at 55° to 70°C

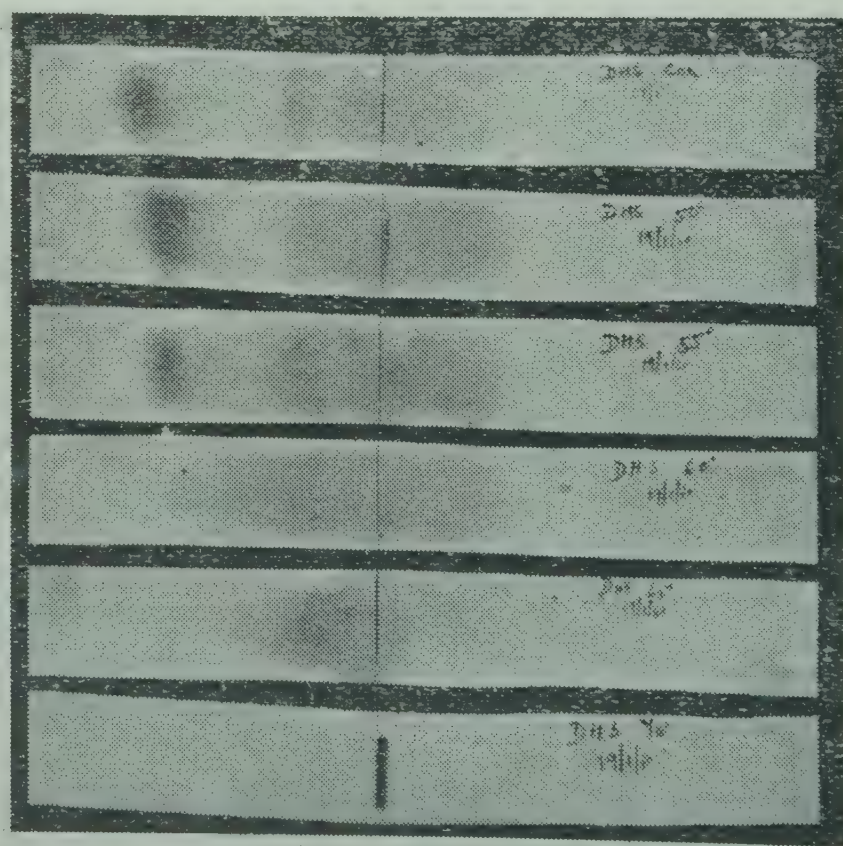


FIG. 4. Paper electrophoresis of diphtheria antitoxic serum heated for 30 min. at 50° to 70°C



TABLE II. *Antitoxic titres of diphtheria antiserum heated at different temperatures*

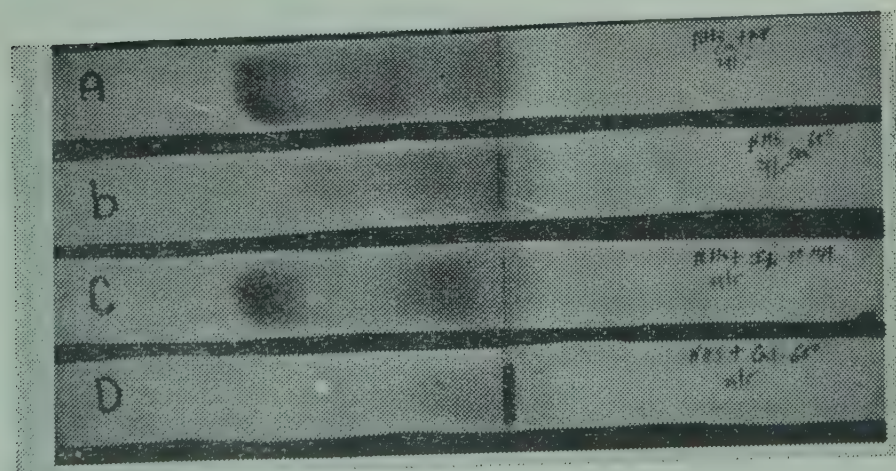
Temperature		Flocculation titre (Time of flocculation in brackets)	Rabbit skin test titre
		I.U.	I.U.
Control	...	150 (20 min.)	250
50°C	...	150 (20 min.)	250
55°C	...	150 (22 min.)	250
60°C	...	150 (80 min.)	250
65°C	...	No flocculation in 4 hr.	150
70°C	...	do	90

It will be seen from Table II that the antitoxic titre has remained about the same till 60°C, after which the antisera do not flocculate with their toxins. However, the *in vivo* test has shown that the antitoxic titre is not lost completely but decreases rapidly when heated above 60°C.

IV. *Effect of glutathione and iodoacetate on the protein interactions in sera:* At this stage of the work it was evident that protein-protein interaction takes place in sera under the influence of heat. The most reactive groups in proteins are known to be the sulphydryl and the disulphide groups. Next in order of reactivity are the free phenolic group of tyrosine and the imidazole group of histidine.

According to Jensen<sup>2,3</sup>, proteins can, under the influence of heat, polymerise or combine with other proteins by a process of sulphydryl-disulphide interchange. It is known that serum albumin and  $\gamma$ -globulin contain both sulphydryl and disulphide groups. It was therefore decided to test the effect of heat on serum proteins in the presence of iodoacetate which combines with sulphydryl groups and of reduced glutathione which can reduce the disulphide groups to sulphydryl groups.

Normal horse serum diluted with an equal volume of water was heated at 60°, 65° and 70°C for 30 minutes in presence of 50 millimoles of iodoacetate and other aliquots were heated at these temperatures with 5 millimoles of reduced glutathione. The samples were subjected to electrophoresis. Figure 5 is a photograph of the electrophoresis strips of serum samples heated at 65°C.



A = Normal horse serum not heated

B = Heated at 65°C

C = Heated at 65°C with iodoacetate

D = Heated with glutathione at 65°C

FIG. 5. Effect of iodoacetate and glutathione on heating of normal horse serum heated for 30 min. at 65°C



It will be seen from Figure 5 that iodoacetate to some extent prevented the interaction of the proteins. On the other hand, glutathione accelerated the combination.

The above work was repeated with antidiphtheria horse sera and the results were similar. The antitoxic titre was more or less the same with iodoacetate or glutathione and preliminary experiments have shown that it remained the same as the controls heated at 60°C for 30 minutes.

## Discussion

Schulz<sup>5</sup> carried out heating experiments with human serum and reported changes in electrophoretic mobility of albumin and also of other globulins. There was no further evidence to suggest a protein-protein combination. The present results based on electrophoresis, as well as on immunoelectrophoresis of heated sera, show clearly the lack of any significant change in the mobility of the proteins. The changes observed are caused by the aggregation of different proteins of sera under the influence of heat. This is clear from the results of experiments on heating of purified albumin which shows no significant change in quantity or mobility, though there is increase in cloudiness and viscosity above 60°C, indicating polymerisation. On the other hand, albumin decreases in amount when heated with other serum proteins indicating its combination with these proteins. This is confirmed by the immunoelectrophoresis experiments. There is evidence in literature that serum albumin polymerises on heating and Jensen<sup>4</sup> is of the opinion that this is due to sulphhydryl-disulfide exchange. It is also known from the literature<sup>6</sup> that human serum albumin has one sulphhydryl group and 16 disulfide groups per molecule and human  $\gamma$ -globulin has an average of 5.8 sulphhydryl groups and 10 disulfide groups per molecule. These groups being the most reactive are likely to bring about polymerisation or copolymerisation with other proteins at higher temperatures.

Recently, Christian<sup>7</sup> has shown that human  $\gamma$ -globulin (commercial Cohn fraction II) aggregates when heated at 56°C for 30 minutes as shown by an increase in the sedimentation constant. Such aggregated  $\gamma$ -globulin has been shown by him to have anti-complementary activity. Ishizaka<sup>8</sup> has also found some alteration in skin-reactive properties of heated  $\gamma$ -globulin. Maurer and Thorpe<sup>9</sup> have recently found a reduced precipitating ability of rabbit antisera to bovine serum albumin when it is heated at 70°C for 10 minutes. Our object is to find out how far the loss in antitoxic activity of immune  $\gamma$ -globulin can be correlated with polymerisation or combination with other proteins such as albumin. Experiments are now in progress to prepare pure anti-diphtheria  $\gamma$ -globulin from hyperimmunized horse sera using DEAE cellulose and to study how its antitoxic activity is affected under different conditions. Preliminary experiments have shown that masking of SH groups of diphtheria antitoxic pseudo-globulin solution with iodoacetate does not markedly reduce its reactivity with diphtheria toxin or its reactivity with its antibody (anti-horse-serum) produced in rabbits. It is, therefore, likely that masking of the sulphhydryl groups may make the serum proteins more stable to heat.

## Summary

1. Effects of heating normal and immune horse serum for 30 minutes at temperatures between 50°C to 70°C and analysis of the heated samples by paper electrophoresis and



immunoelectrophoresis has shown that protein-protein interactions take place. The albumin was found to be particularly active in combining with other serum proteins.

2. The protein-protein interactions could be accelerated by the presence of reduced glutathione and inhibited by iodoacetate. This suggests that the active group taking part in these reactions is the sulphydryl group of the serum proteins.

3. The bearing of the results on deterioration of immune sera during storage is discussed.

### Acknowledgment

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### Discussion

Q. Does dialysis stabilise the antitoxic activity?

A. No.

Q. Is it not possible that groups other than the sulphydryls may be involved in the observed interactions? There may also be a factor in serum which slowly reacts with and blocks the active groups of the antitoxic substance thus causing loss in potency. Reduced glutathione may combine with such a factor.

A. While the experimental results presented do not rule out such possibilities, the stabilising effect of reduced glutathione and the opposite effect of iodoacetate strongly implicate SH- groups in these interactions.

Q. Has the effect of specific SH- reacting agents like parachloromercuribenzoate (PCMB) been studied?

A. It is proposed to investigate the effect of PCMB in further work on the problem.



# EFFECT OF ACIDS ON PROTEINS

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This investigation had its origin in an accidental finding that seminal plasma proteins, after precipitation with 5 per cent trichloroacetic acid (TCA) and washing twice with sodium chloride, could be dissolved, almost completely, in distilled water. Since denaturation of proteins is usually understood to result in a loss of solubility, it appeared that a major portion of the seminal plasma proteins was not denatured by 5 per cent TCA, although the proteins were precipitated. Our observations on the effect of TCA on seminal plasma proteins are recorded in this paper.

Since seminal plasma is analogous to blood plasma, we have also studied the action of various acids on blood plasma proteids.

It has been recently shown that the architecture of a protein molecule as it occurs in the native state, is not essential for its enzymic activity. Thus chymotrypsin<sup>1</sup>, trypsin<sup>2</sup>, papain<sup>3</sup>, and ribonuclease<sup>4</sup> have been shown to retain their enzymic activity even after considerable degradation and consequent reduction in molecular weight. The antigenic properties of bovine serum albumin (BSA) are unaltered even after removal of a large peptide by the action of trypsin<sup>5</sup>. Ovalbumin spread in the form of 9 Å<sup>o</sup> thick monolayers, under which condition the molecule should be drastically modified, has been shown to retain the ability to combine with its specific antibody<sup>6</sup>.

## Experimental Results

*Effect of TCA on seminal plasma proteins:* The seminal plasma was separated from spermatozoa by centrifugation of semen diluted with Krebs-Ringer bi-carbonate buffer, at 3,000 r.p.m. for 5 minutes. The diluted seminal plasma was treated with an equal volume of 10 per cent TCA at room temperature and the precipitate washed twice, each with 5 per cent TCA and 5 per cent sodium chloride. The sodium chloride washings were turbid. The residue was extracted with water. Almost the whole of the residue went into solution\*.

The material in solution was isolated by freeze-drying. Its absorption spectrum was typical of proteins. A solution containing 1 mg. of the material per ml. gave an optical density of approximately 1.15 at 280 mμ in a one cm. cell, a value within the range usually obtained for proteins. The above material gave a positive biuret test and was found to contain 13-14 per cent nitrogen as determined by the micro-Kjeldahl method, further confirming that it was protein. The material was not coagulated when an aqueous solution was boiled for 5 minutes, probably due to the presence of traces of TCA. The presence of TCA (0.1-0.9 per cent) was found to prevent heat coagulation of blood plasma proteins also.

An aqueous solution of the above protein material, in which protein concentration was determined by measurement of optical density at 280 mμ, was dialysed against frequent changes of distilled water at 3°C for approximately 72 hours to remove adhering salts. The dialysis was stopped when the dialysate did not give a positive test for chloride.

\* As will be discussed later, the presence of small amount of sodium chloride, left over after sodium chloride washing of the TCA precipitate, made possible the complete extraction of the material with water.



Dialysis resulted in a considerable loss of material absorbing at 280  $m\mu$ . Part of the non-dialysable fraction got precipitated, which was shown to be due to removal of salts. The seminal plasma proteins, after precipitation with TCA, could thus be resolved into three fractions: (i) Fraction A, which is dialysable; (ii) Fraction B, which is non-dialysable and water-insoluble and (iii) Fraction C, which is non-dialysable but water-soluble. The results of fractionation of seminal plasma proteins of two bulls of different breed (one, a buffalo bull) and one goat are given in Table I.

TABLE I

*Fractionation of TCA precipitated seminal plasma protein by dialysis*

All data are for seminal plasma derived from 1 ml. of semen. Protein was estimated by measurement of optical density at 280  $m\mu$ , except in the case of the residue in column 4, which was weighed, and fraction A, which was calculated by difference (Z-B-C). An optical density of 1.25 in a 1 cm. cell was presumed to correspond to 1 mg. of protein. All weights are in mg. Percentages are calculated on column Z.

Animal	Wt. of seminal plasma from 1 ml. of semen*	TCA precipitated material after washing with 5% sodium chloride		Dialysable portion of fraction Z (Fraction A)	Non-dialysable portion of fraction Z precipitated on dialysis (Fraction B)	Non-dialysable portion of fraction Z not precipitated on dialysis (Fraction C)	Total non-dialysable portion Fractions (B + C)
		Amount of protein extracted by water (Fraction Z)	Residue after extracted by water				
Kerry bull (5 experiments)	80-103	40-60	0.5-1.5	10-16 (25-30%)	22-40 (50-64%)	4-8 (11-13%)	30-45 (63-73%)
Buffalo bull (4 experiments)	45- 60	23-34	0.4-1.0	6-9 (26-29%)	14-22 (61-66%)	3-4 (12-15%)	17-26 (72-80%)
Deoni bull (4 experiments)	75-100	50-65	0.7-1.5	17-22 (31-36%)	30-35 (57-61%)	5-8 ( 9-12%)	37-43 (68-73%)
Jamnapari goat (2 experiments)	120-155	70-80	1.0-1.9	...	...	...	...

\*Calculated by difference (dry weight of 1 ml. semen—dry weight of spermatozoa from 1 ml. semen).

*Fraction A:* This fraction represented 25-36 per cent of the total proteins in solution at the beginning of the dialysis. This material must be of sufficiently high molecular weight to be precipitated by TCA but yet small enough to pass through the cellophane membrane. Presence in seminal plasma of a protein-like material which passes readily through a semi-permeable membrane, has been demonstrated<sup>7</sup>. However, this material has been reported not to be precipitated by TCA<sup>8</sup> and, therefore, is unlikely to be identical with our fraction A. Since semen was stored for 2-3 hours after collection before working it up, it is possible that fraction A may represent polypeptides obtained as a result of proteolysis of seminal plasma proteins<sup>9</sup>.

*Fraction B:* This fraction is soluble in 0.9 per cent sodium chloride and was precipitated on dialysis due to removal of sodium chloride; the presence of small amount of this salt in the sodium chloride washed TCA precipitate made possible initially the extraction of this protein fraction by water. A solution of this material in 0.9 per cent sodium chloride was not coagulated on boiling for 5 minutes. Fraction B represented 45-57 per cent of the total TCA precipitated protein which was extractable by water after sodium chloride washing. The amino acid composition of this fraction (Table II)



TABLE II. *Amino acid composition of Fraction B derived from seminal plasma protein*

Amino acid		Content (g./100g protein)
Glutamic acid	...	5.71
Aspartic acid	...	7.72
Lysine	...	10.14
Arginine	...	3.6
Phenylalanine	...	6.61
Tyrosine	...	6.55
Serine	...	3.75
Theronine	...	5.76
Methionine	...	11.94
Cystine	...	6.07
Alanine	...	1.66
Glycine	...	0.67
Valine	...	3.81
Leucine	}	8.05
Isoleucine		
Tryptophan*	...	...
Proline*	...	...
Total	...	82.0

\* Not estimated

was determined by quantitative two dimensional paper chromatography, using butanol: acetic acid: water (4:1:5), followed by phenol: meta-cresol (1:2) containing 16.7 ml. of pH 8.3 borate buffer per 100 ml. of the phenolic mixture, as the developing solvents<sup>10</sup>. It contained all naturally occurring amino acids, except histidine. The salt solubility of this fraction suggests its globunoid nature. A solution of this protein in 0.9 per cent sodium chloride was precipitated by yeast ribonucleic acid.

*Fraction C:* This fraction is soluble in water as well as in 0.9 per cent sodium chloride. It represented 5-12 per cent of the TCA precipitated protein extracted by water after sodium chloride washing. This protein may be albuminoid in nature.

*Effect of TCA and other acids on blood plasma proteins:* Citrated blood was collected from two groups of rabbits and several adult men and the plasma separated by centrifugation (2,500 r.p.m. for 10 minutes). The total protein in the plasma was estimated (a) by determining the nitrogen content of a known quantity of plasma by micro-Kjeldahl method and multiplying the nitrogen value by 6.5; (b) by determining the dry weight of the residue obtained on precipitation of the plasma with an equal volume of 10 per cent TCA, followed by removal of nucleic acids and lipids; and (c) by measurement of optical density of the hundred fold diluted plasma at 280 m $\mu$ . The first two of the above methods gave identical values for the protein content in a large number of samples of rabbit and human plasma (for examples, see Table III). The value obtained by optical density measurement was generally a little higher than the above values in the case of rabbits, but lower in the case of humans.

In one experiment, rabbit blood plasma proteins were precipitated with an equal volume of 10 per cent TCA and washed twice each with 5 per cent TCA and 5 per cent sodium chloride. The residue was extracted exhaustively with water. About 80 per cent



TABLE III. *Solubility of rabbit blood plasma protein after TCA precipitation*

For details of isolation of the soluble and insoluble portion of the TCA precipitated protein in experiments I and II, see text. In both the experiments, 3 ml. of plasma were used. The blood was withdrawn from the same animal at an interval of approximately two weeks. An optical density of 1.25 was presumed to correspond to 1 mg. of protein.

	Total protein (mg.)			TCA precipitated protein (mg.)		
	By dry weight determination	By O.D. measurement	By nitrogen estimation	Insoluble portion	Soluble portion	
	(a)	(b)	(c)	By dry weight determination	By O.D. measurement	Calculated (O.D. $\times$ a/b)
Experiment I ...	194.4	176.7	189.1	35	140	154
Experiment II ...	190.0	176.4	188.3	10	169	181

of the residue went into solution. However, since some denaturation seemed likely due to repeated extraction extending over several hours, in subsequent experiments the residue after washing with sodium chloride was suspended in distilled water in a cellophane tube and dialysed against frequent changes of distilled water at 3°C for approximately 72 hours. At the end of this period, the dialysate gave a negative test for chloride. A major portion (about 95 per cent) of the plasma proteins, including most of the globulins, went into solution. The solution was slightly opalescent, which may be due to the presence of some protein in an aggregated or a colloidal form. This may also explain the comparatively higher yield of protein obtained in this way, in comparison with the yield obtained after initial exhaustive extraction with water. After dialysis, the residue was separated from the solubilised material by centrifugation at 4000 r.p.m. for 10 minutes. The protein in the supernatant was estimated by measurement of optical density at 280 m $\mu$ . The residue was freeze-dried and weighed. The results of one experiment (Experiment I) in which the TCA precipitated proteins, after washing with sodium chloride, were extracted exhaustively with water before dialysis, and of another experiment (Experiment II) in which the TCA precipitated material was dialysed directly after washing with sodium chloride, are given in Table III.

Since from the above observations it appeared that precipitation of plasma proteins by 5 per cent TCA does not render the proteins insoluble (on the contrary, such treatment appeared to solubilise globulins in water), the action of per-chloric hydrochloric and nitric acids on plasma proteins was studied. The final concentration of these acids for precipitation was also 5 per cent. Perchloric acid, like TCA, precipitated plasma proteins completely. With hydrochloric and nitric acids the precipitation was incomplete. After removal of the precipitate (HCl ppt. I and HNO<sub>3</sub> ppt. I) by centrifugation, the rest of the protein in the supernatants could be precipitated with TCA (HCl ppt. II and HNO<sub>3</sub> ppt. II). The protein precipitated with TCA and PCA, HCl ppts. I and II, and HNO<sub>3</sub> ppts. I and II were removed by centrifugation and dialysed against frequent changes of distilled water for 72 hours at 3°C. In each case, the residue (Fraction R) after dialysis was separated from the dissolved proteins (Fraction S) by centrifugation and extracted with 0.9 per cent sodium chloride. The sodium chloride insoluble portion (Fraction R<sub>1</sub>) of the residue was washed and weighed and the protein in the sodium chloride extract



(Fraction  $R_2$ ) estimated by measurement of optical density. The protein in aqueous solution (Fraction S), which was not precipitated on dialysis for three days, was further dialysed for six days and the precipitated material (Fraction SR) removed, freeze-dried, weighed and extracted with 0.9 per cent sodium chloride to give an insoluble and a soluble fraction (Fractions  $SR_1$  and  $SR_2$  respectively). Fraction  $SR_2$  was estimated by measurement of optical density. The protein in Fraction  $SR_1$  was arrived at by difference ( $SR - SR_2$ ). The protein in solution after the second dialysis (Fraction SS) was estimated by measurement of optical density. Further dialysis of the soluble (SS) fraction did not

TABLE IV. *Fractionation of acid precipitated rabbit blood plasma protein by dialysis*

The fractionation was done in each case with 1 ml. of plasma containing 61.1 mg. of protein based on nitrogen determination (Experiment A in the text). An optical density of 1.25 was presumed to correspond to 1 mg. of protein.

Material		Protein in solution after dialysis (Fraction S)	Protein precipitated on dialysis (Fraction R)		Total protein (Fractions S + $R_1$ + $R_2$ )	Sum of all fraction derived from one acid
			Protein insoluble in 0.9% sodium chloride (Fraction $R_1$ )	Protein soluble in 0.9% sodium chloride (Fraction $R_2$ )		
HCl ppt. I	...	43	mg. 2.1	1.4	46.5	} 60.3
HCl ppt. II	...	5.4	0.4	8	13.8	
HNO <sub>3</sub> ppt. I	...	46	0.4	4.5	50.9	} 59.0
HNO <sub>3</sub> ppt. II	...	4.6	0.3	3.2	8.1	
PCA ppt.	...	49	1.7	8.5	59.2	59.2
TCA ppt.	...	55	1.1	3.0	59.1	59.1

TABLE V

*Dialysis of Fraction S*

For details, see text. An optical density of 1.25 was presumed to correspond to 1 mg. of protein.

Material	Protein in Fraction S before dialysis	Protein insoluble after dialysis (Fraction SS)	Protein pptd. on dialysis (Fraction SR)		Total protein (Fractions SS + $SR_1$ + $SR_2$ )	Sum of all fractions divided from one acid
			Protein insoluble in 0.9% sodium chloride (Fraction $SR_1$ )	Protein soluble in 0.9% sodium chloride (Fraction $S_2$ )		
			mg			
HCl ppt. I	43	32	5.8	3.2	41	} 46
HCl ppt. II	5.4	4	0.3	0.7	5	
HNO <sub>3</sub> ppt. I	46	34	8	2	44	} 48
HNO <sub>3</sub> ppt. II	4.6	1	0.4	2.6	4	
PCA ppt.	49	34	8	6	48	48
TCA ppt.	55	52	1.8	1.2	55	55



yield any more salt soluble protein, but part of the soluble portion was rendered insoluble. Results of quantitative estimation of each of the fractions derived from rabbit blood plasma protein precipitated with various acids, in a typical experiment, are given in Tables IV and V.

Since dialysis of acid precipitated plasma proteins yielded a water-soluble and a salt-soluble fraction, it appeared likely that these fractions correspond to conventional albumin and globulin fractions of the plasma. The albumin and globulin content of rabbit blood plasma was, therefore, determined in the usual manner by ammonium sulphate fractionation. The results of two representative experiments with rabbit plasma are given in Table VI.

TABLE VI

*Ammonium sulphate fractionation of rabbit blood plasma protein*

Six ml. of plasma was taken for fractionation in each experiment. The precipitate was dialysed against distilled water for 2-3 days; the protein in solution was estimated by measurement of optical density, and the residue was weighed. An optical density of 1.25 was presumed to correspond to 1 mg. of protein.

Protein fraction	Percentage saturation with respect to ammonium sulphate	AMOUNT OF PROTEIN PRECIPITATED			
		mg.		Percentage of total protein	
		Experiment A*	Experiment B†	Experiment A*	Experiment B†
Fibrinogen ...	26	12	8	3.1	2.7
Euglobulin ...	33	45	35.4	11.7	12.2
Pseudoglobulin ...	46	37.5	31.2	9.8	10.8
Albumin ...	100	289	216	75.3	74.5

\*The same batch of animals was used in this experiment as in the experiment described in Table IV and V.

†The animals used in this experiment were much younger than the animals used in experiment A.

It will be seen that, on dialysis, the acid precipitated plasma protein could be resolved into three fractions: Fraction I, a water-soluble fraction (Fraction SS); Fraction II, a salt-soluble fraction (Fractions  $R_2$  plus  $SR_2$ ) and Fraction III, an insoluble fraction (Fractions  $R_1$  plus  $SR_1$ ). Recovery data on these fractions calculated from Table IV and V (Experiment A), and from another experiment (Experiment B) in which the dialysis was carried out for 9 days at a stretch, are given in Table VII.

*Fractions I and III:* The total amount of protein in Fraction I, which would appear to consist of albumin, is the same in the case of hydrochloric, nitric and per-chloric acid treatment of plasma. In the case of TCA, as has already been discussed, a lot more protein is held in solution. Excellent correspondence was obtained between the albumin content of plasma (74-76 per cent of the total plasma protein, Table VI) as determined by ammonium sulphate fractionation, and the total of Fractions I and III (73-78 per cent of the total plasma protein, Table VII), in both the experiments. Since some denaturation of albumin on prolonged dialysis is possible, it appears that the insoluble fraction III may consist mainly of denatured albumin. This is supported by the observation that further dialysis of fraction SS (Experiment A) resulted in progressive loss of solubility without precipitation of any more salt-soluble protein.



The results in Table VII suggest the presence of at least three different albumin components: one ( $C_1$ ) which is precipitated with both hydrochloric and nitric acid, the other ( $C_2$ ) which is precipitated with TCA after removal of mineral acid precipitates in either case and the third ( $C_3$ ) which is precipitated with TCA after removal of mineral acid precipitate only in the case of hydrochloric acid (in the case of nitric acid this component is

TABLE VII. *Summary of the results of two experiments on the fractionation of acid precipitated plasma proteins*

Data on Experiment A are taken from Tables IV and V. For Experiment B, see text. One and two ml. of plasma, containing 61.1 mg. and 90.8 mg. of protein (based on nitrogen determination), were used in Experiments A and B respectively. Figures in parenthesis refer to the protein content of mineral acid precipitates I and II respectively (see text).

Material		Water soluble protein (Fraction I)		Salt soluble protein (Fraction II)		Insoluble protein (Fraction III)		Fraction I + III Percentage of total protein
		Milligrams	Percentage of total protein	Milligrams	Percentage of total protein	Milligrams	Percentage of total protein	
HCl ppts. I & II	Expt. A	36 (32 + 4)	62.2	13.3 (4.6 + 8.7)	23.0	8.6 (7.9 + 0.7)	14.8	77.0
	Expt. B	55 (49 + 6)	65.5	22.0 (15 + 7)	26.2	7 (4 + 3)	8.3	73.8
HNO <sub>3</sub> ppts. I & II	Expt. A	35 (34 + 1)	62.1	12.3 (6.3 + 6.0)	21.8	9.1 (8.4 + 0.7)	16.1	78.2
	Expt. B	51.5 (50 + 1.5)	60.2	23.0 (22 + 1)	26.9	11 (4 + 7)	12.9	73.1
PCA ppt. ...	Expt. A	34	58.5	14.5	24.9	9.7	16.6	75.1
	Expt. B	52.5	59.3	24.0	27.1	12	13.6	72.9
TCA ppt. ...	Expt. A	52	88.0	4.2	7.1	2.9	4.9	92.9
	Expt. B	84	96.0	1.0	1.1	2.5	2.9	98.9

probably precipitated along with  $C_1$ ). The components  $C_1$ ,  $C_2$  and  $C_3$  represent on an average 87.9, 2.8 and 9.3 per cent of the total proteins in the water-soluble Fraction I. One of these minor components may be the conventional  $\alpha_2$ -albumin fraction.

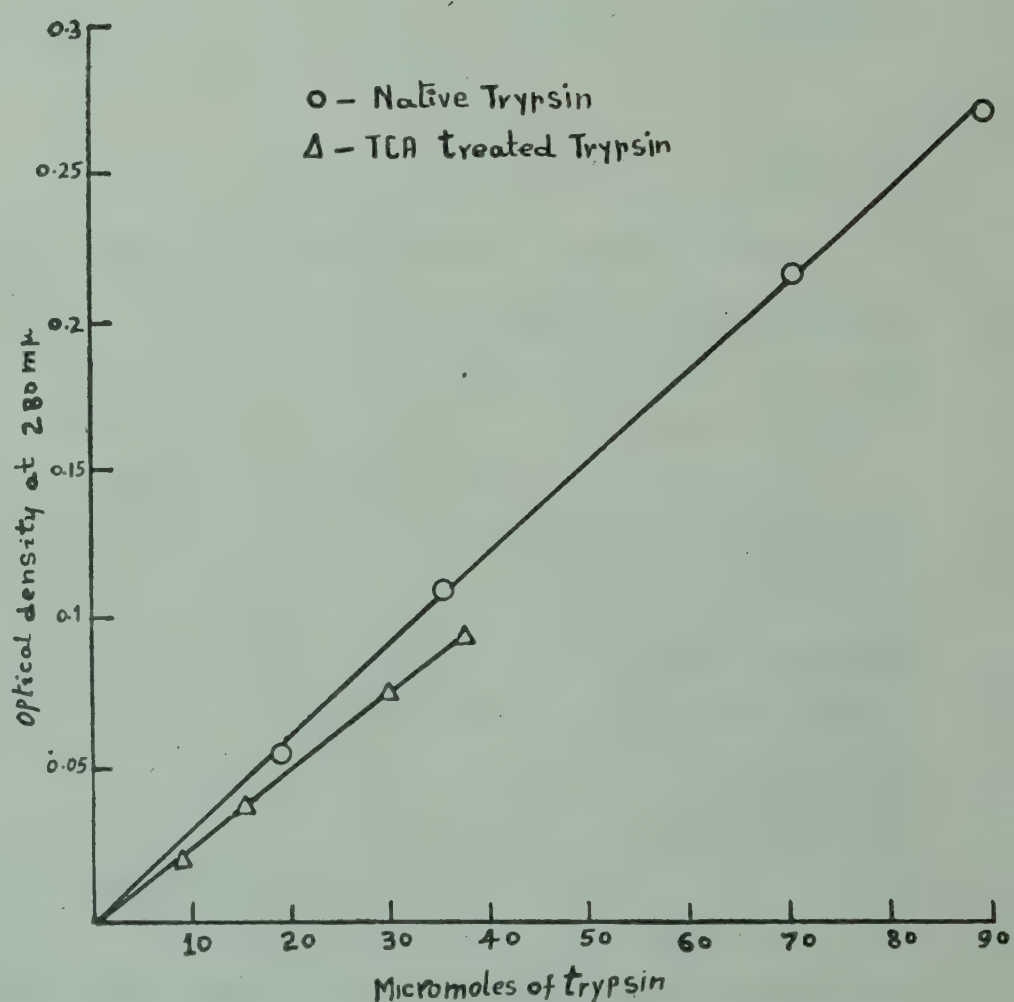
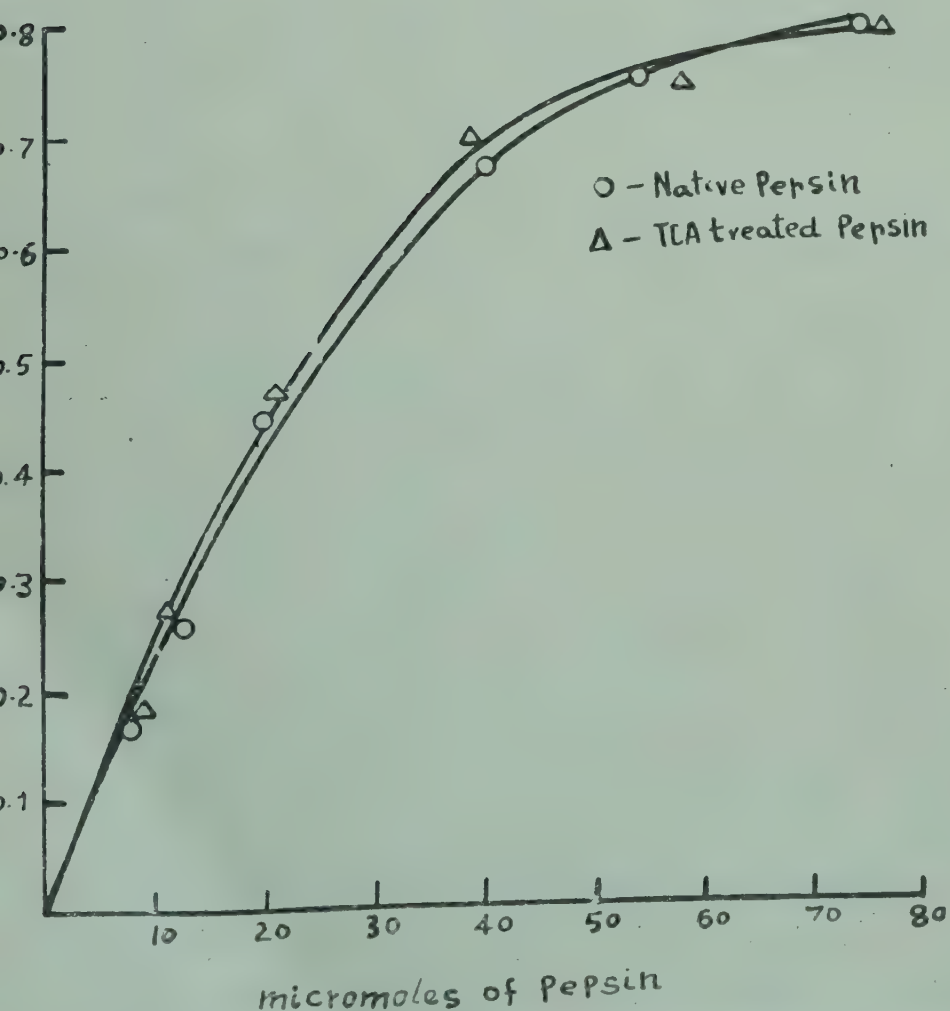
*Fraction II:* The globulin, pseudoglobulin and fibrinogen content of rabbit blood plasma as determined by ammonium sulphate fractionation was 24-26 per cent. The salt-soluble fraction II (Table VI) derived from hydrochloric acid, nitric acid or PCA treatment of plasma represented between 22-27 per cent of the total plasma proteins. It would therefore appear that fraction II consists of globulins (normal and pseudo-) and possibly fibrinogen, which are all salt-soluble. It is difficult to interpret the fractionation of the salt-soluble components that we have obtained with hydrochloric and nitric acids. The HCl ppts. I and II, and the HNO<sub>3</sub> ppts. I and II, all contained salt-soluble proteins, the content of which appeared to be different in the precipitates I and II derived from different mineral acids. Moreover, there was also a difference in the relative proportion of fraction II in these precipitates in different experiments.



**Effect of TCA on crystalline enzymes:** An aqueous solution of pepsin (3 mg./ml.) or of trypsin (3 mg./ml.) was treated with an equal volume of 10 per cent TCA at room temperature and the material allowed to stand for 5 minutes. Complete precipitation of trypsin occurred, but pepsin remained in solution. Trichloroacetic acid from the pepsin solution and from precipitated trypsin was removed by dialysis at 4°C against frequent changes of Clark's HCl/KCl buffer (pH 1.8) and Sorenson's phosphate buffer (pH 7.6) respectively. No precipitate was obtained in either case. After about 48 hours' dialysis, the enzyme activity of the solution was compared with that of the native enzyme (dialysed for the same period against the buffer). Pepsin and trypsin activities were determined by modifications of the method of Anson<sup>11</sup> and Kunitz<sup>12</sup> respectively, the extent of degradation of casein being determined by measurement of the optical density of the acid-soluble material at 280 m $\mu$ , after removal of the undegraded substrate by precipitation with TCA. The results are shown in Fig. 1 and 2. The enzyme activity of pepsin was completely retained after treatment with TCA. The activity of trypsin was also recovered almost entirely after TCA treatment.

## Discussion

Recently, Gonashvili<sup>13</sup> suggested that the mechanism of coagulation of proteins at a high TCA concentration is analogous to the action of strong mineral acids. Since the action of the latter acids on proteins is believed to involve irreversible denaturation, the action of TCA also would be expected to result in an alteration of the physical and biological properties of proteins. However, Steinrauf<sup>14</sup>, from his studies on the properties of bovine serum albumin (BSA), found that the intrinsic viscosity, specific optical rotation, and sedimentation constant of BSA in concentrated acetic acid is different from that of the same preparation in aqueous acetate buffer; in spite of this, BSA could be recovered apparently



1. Enzyme activity of native and TCA treated pepsin. FIG. 2. Enzyme activity of native and TCA treated trypsin.



unchanged from acetic acid by lyophilization, suggesting that certain changes in the tertiary structure of proteins may not affect their physical and biological properties, like solubility and enzyme activity. From the results reported in this paper it appears that although acids may precipitate proteins, in some cases and under certain conditions, the protein may not be denatured, denaturation being considered to involve loss of biological activity, usually accompanied by a loss of solubility. The possibility of reversible denaturation of the proteins studied in this paper, on treatment with acid, although unlikely, should however, not be ruled out. Since on treatment with 5 per cent acid, an alteration in the three-dimensional structure of proteins is most likely due to break of hydrogen bonds, it appears that a change in the tertiary structure of a protein does not necessarily lead to a loss of biological activity.

Coagulation of proteins by high concentrations of acids may be distinguishable from denaturation and may involve changes in protein structure which are separate from changes which occur during denaturation. Such changes may not affect the enzymatic activity of proteins, in contrast to the changes brought about during denaturation. Further information on the above issue is hoped to be gained by determination of electrophoretic mobility, isoelectric point, viscosity, molecular weight, and specific rotation of those proteins, the solubility and biological properties of which are not affected by precipitation with acids.

## Summary

The effect of acids at a concentration of 5 per cent on denaturation of seminal plasma and blood plasma proteins, and crystalline pepsin and trypsin was studied. The proteins of seminal plasma precipitated by trichloroacetic acid (TCA) were apparently not denatured. The TCA precipitated proteins could be resolved into three fractions, one of which was dialysable. The major portion of the non-dialysable protein material was soluble in dilute salt solution, indicating its globunoid nature. The rest of the non-dialysable protein material was soluble in water.

Trichloroacetic, perchloric (PCA), hydrochloric and nitric acids had apparently no effect on the solubility characteristics of the various fractions of rabbit blood plasma proteins. It appeared that traces of TCA could firmly bind globulins and prevent their precipitation from salt solutions on dialysis. Traces of TCA or of other acids could also prevent heat coagulation of plasma proteins.

Treatment of pepsin and trypsin with 5 per cent TCA did not affect their enzymatic activity, although a change in the tertiary structure of these proteins would probably be inevitable as a result of such treatment.

The observations reported in this paper suggest that modification of the tertiary structure of a protein may not necessarily result in the loss of biological activity.

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## Discussion

- Q. Certain enzymes—trypsin, chymotrypsin, papain and ribonuclease—are known to retain their full activity even after large peptide residues are split off from their molecules. What are the special features of the results now presented?
- A. The present results primarily concern certain blood and seminal plasma proteins under comparatively mild conditions which are expected to bring about changes only in the tertiary structure of proteins.
- Q. Was the TCA-filtrate examined for peptides and amino acids in the case of plasma proteins?
- A. No.
- Q. Did increase in TCA concentration affect the results?
- A. The pattern was the same.
- Q. Apart from solubility behaviour what other properties of the acid treated proteins have been studied for purposes of comparison?
- A. It is proposed to examine their biological (immunological) and other properties.
- Q. In the case of the enzymes studied, was any activity found in the TCA-filtrate?
- A. The filtrates have not been thus examined particularly because of the suspected interference of the acid present in large concentration.
- Q. How could one say that the residual protein after acid treatment is the same as the original?
- A. No, we cannot say that. Only after completing the proposed biological tests and other physico-chemical studies would it be possible to say in what respects the treated proteins are similar to the native proteins.



# REACTIONS BETWEEN FORMALDEHYDE AND PEPTONE

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Reactions between formaldehyde and proteins are employed in the production of plastics, in tanning leather and in the preparation of toxoids. It is believed that some of these reactions are 'reversible' while others are 'irreversible'. Some reactions taking place between formaldehyde vapour and liquid and peptone studied in our laboratories are described in this paper.

## Experimental Materials and Methods

*Peptones:* Two commercial brands of peptone A and B were used.

*Chromotropic acid and Dimedone* used for the estimation of formaldehyde were of commercial quality (B.D.H.).

*Chromotropic acid reagent*<sup>1</sup> 0.2 per cent solution of the substance in a 2:1 sulphuric acid solution.

*Vorlander's reagent*<sup>2,3</sup> (Dimedone solution): 0.2 per cent solution of dimedone in McIlvaine buffer of pH 7.4.

*Determination of formaldehyde by the chromotropic acid reagent:* One ml of test solution containing about 2-3 mg of formaldehyde was mixed with 9 ml of chromotropic acid reagent and heated in a boiling water bath for 30 min. The purple colour developed was measured in a Spekker photoelectric absorptiometer using a combination of blue and orange filters and as blank a solution obtained by repeating the process using 1 ml of distilled water in the place of test solution.

*Determination of bound formaldehyde by Vorlander's reagent (Method I):* 2 ml of test solution containing about 40-50 mg. of formaldehyde were mixed with 2 ml. of Vorlander's reagent and kept at 37° for 30 min. to remove free formaldehyde. Formaldehyde content in 1 ml. of this mixture was estimated by the chromotropic acid reagent as described above.

*Determination of bound formaldehyde by formol titration (Method II):* 10 ml of the solution containing about 100 mg. of peptone were titrated with carbonate-free 0.1 N and alkali to a pink colour with phenolphthalein. 10 ml. of neutral formalin was then added and the titration again continued to a pink colour. This volume of alkali was noted as formol titre and was calculated as number of ml of decinormal alkali required by 1 gram of original peptone and referred to as formol titration value (F.T.V.).



From F.T.V.s of the original peptones and reaction products of peptone and formaldehyde vapour (described below), the bound formaldehyde was calculated as in the following example:

F.T.V. of original peptone A15.4

F.T.V. of the reaction product of peptone and formaldehyde vapour4.3

Difference.11.1 ml. of 0.1 N. NaOH.

But 1 ml. 0.1 N NaOH in the formol titration = 3 mg. HCHO.

11.1 ml. 0.1 N NaOH = 33.3 mg. HCHO.

Similarly peptone B was found to contain 25.2 mg. of HCHO.

Results

*Reaction between formaldehyde vapour and peptone:* Peptones A and B were made to absorb formaldehyde vapour by exposing about 2 grams of peptone (accurately weighed) in a desiccator containing 100 ml. of formalin for 4 days. By this time, the substance became a paste and was transferred to a second desiccator containing phosphorous pentoxide and dried under reduced pressure for a week. The resulting dry masses were dissolved in water and the solutions stored at room temperature.

*Formaldehyde content:* When estimated by the chromotropic acid reagent, each gram of peptone A and B was found to absorb 93.3 and 59.7 mg. of HCHO during exposure to formaldehyde vapour.

*Bound formaldehyde content:* Bound formaldehyde content of these products was determined by chromotropic acid method after Vorlander's reaction (Method I) and formol titration (Method II) and are given in Table I. The bound formaldehyde contents of the solutions on storage are also shown in Table I.

*Reactions taking place between formaldehyde solution and peptone:* A.R. formaldehyde solution equivalent to 94.0 and 60.0 mg. of HCHO was added to 100 ml. of 1 per cent solutions of peptone A and B. The solutions were shaken well and stored at room temperature for 7 days. The bound formaldehyde contents of these solutions were determined as described above and are given in Table I.

TABLE I. Variation of bound formaldehyde content on storage (mg per gram of peptone)

No. of days of storage of solution	Reaction products of formaldehyde vapour and peptone A and B				Reaction products of formaldehyde solution and peptones A and B			
	Peptone-A		Peptone-B		Peptone-A		Peptone-B	
	Method I	Method II	Method I	Method II	Method I	Method II	Method I	Method II
0	27.7	33.3	25.9	25.2	2.6	2.4	1.1	1.5
1	23.1	29.4	22.1	21.9	...	...	...	...
2	21.5	25.8	22.5	21.9	2.6	2.4	3.4	1.5
3	21.0	24.0	19.2	20.1	...	...	...	...
4	20.7	24.0	19.8	18.3	3.0	3.0	3.7	2.4
5	20.1	22.2	18.2	18.3	...	...	...	...
6	20.1	22.2	18.4	18.3	2.7	3.0	3.4	3.0



From the results shown in the Table I, it was found that the bound formaldehyde contents determined by methods I and II agreed closely. They were different when formaldehyde vapour was allowed to react with peptone and when an equivalent quantity of formaldehyde solution was added to peptone. The bound formaldehyde contents of the solutions of the products of formaldehyde vapour and peptone were found to decrease on storage while those of formaldehyde solution and peptone were either constant or slightly increased.

## Discussion

When exposed to formaldehyde vapour, about 60-90 mg. of formaldehyde was absorbed by each gram of peptone. The formaldehyde so absorbed seems to be firmly attached to the different reactive groups of the peptone molecules since it could not be removed even by drying under reduced pressure over phosphorus pentoxide. Determination of bound formaldehyde by two different methods indicated that about 25-35 mg. of formaldehyde was bound in a firm manner. According to French and Edsall,<sup>4</sup> under the conditions employed in formol titrations, the amount of alkali consumed is a direct measure of the free amino groups present in the protein. The differences noted in the F.T.Vs in the case of original peptones and products of peptone and formaldehyde vapour seem to be due to formaldehyde binding to some of the free amino groups of the peptone molecule. Bound formaldehyde calculated by Method-II may be considered to be bound to the amine groups and it seems that part of this formaldehyde is reversibly released on storage of the solutions.

But when a small quantity of formaldehyde solution (equal to that absorbed by peptones when exposed to formaldehyde vapour) was added to peptone solutions, the bound formaldehyde content was found to be between 1-3 mg. only. These low values indicate that a very small quantity is bound to the amino groups under these conditions. This finding is in conformity with the results reported by Hewitt<sup>5</sup> who found that most of the amino groups of the diphtheria toxin were free when it was incubated with 0.3-0.4 per cent formalin. He found that formaldehyde combined with groups other than the amino groups of the diphtheria toxin and was bound irreversibly.

From the present work, it appears that formaldehyde binds differently to peptone when allowed to react as vapour. Probably as vapour, it preferentially binds to amino groups and part of the formaldehyde so bound is released in solution.

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# COMPARATIVE STUDY OF ALKALI AND SWAN'S REAGENT IN THE EXTRACTION OF PROTEINS FROM PLANT MATERIALS

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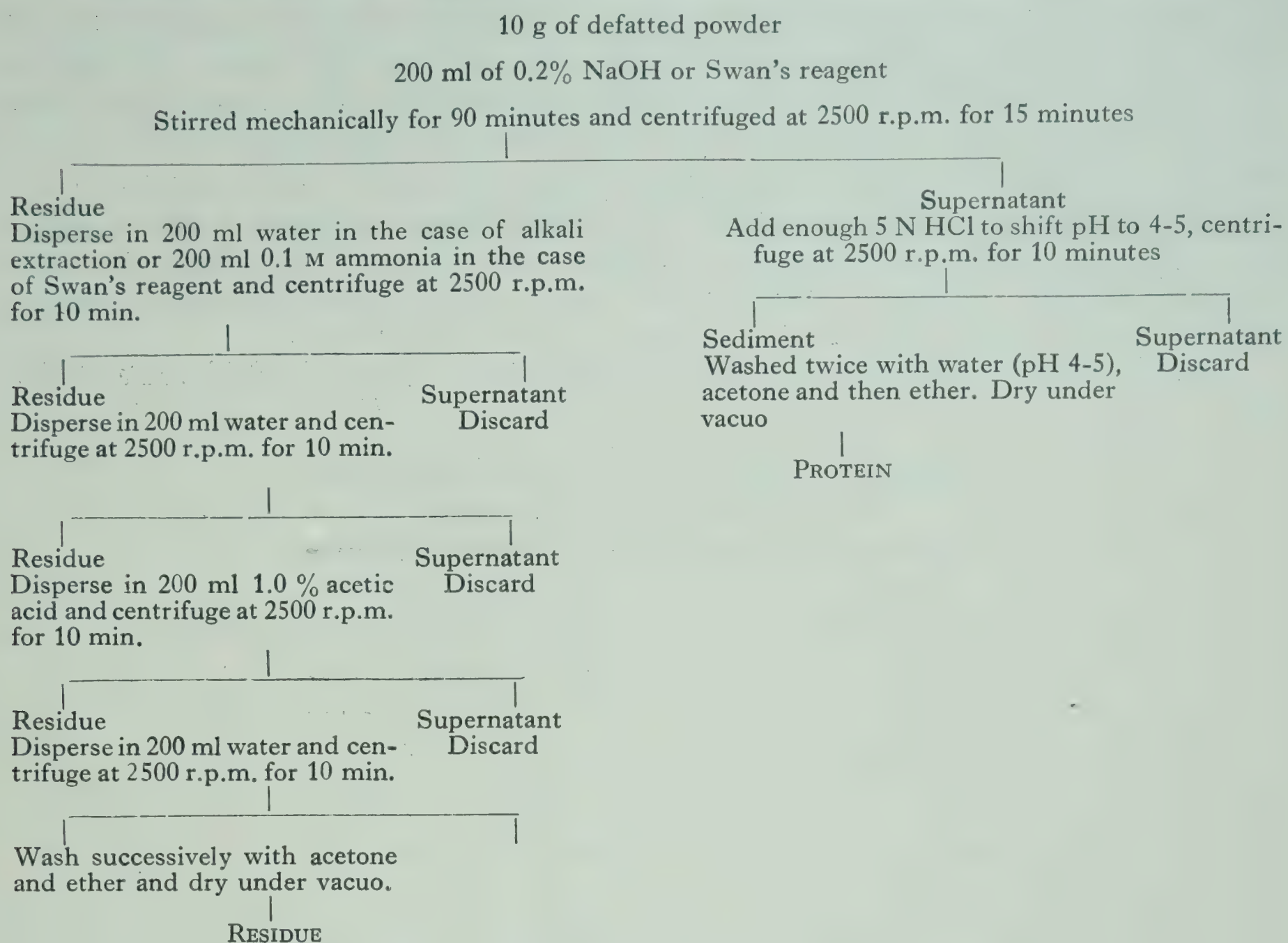
Swan has recently introduced a new reagent for the solubilization of keratin from wool and, under optimal conditions, 85 to 90 per cent of the wool is dissolved by this reagent within a reaction time of 2 to 6 days. The reagent is made up of cuprammonium hydroxide in which sodium sulphite and urea are dissolved to give effective concentrations of 0.05 M and 8 M respectively and the reagent as employed by Swan is 0.02 M with respect to cuprammonium hydroxide. The function of urea is apparently to swell the wool and allow efficient penetration of the extracting reagent. McDermott and Pace have reported total extraction of proteins from wheat flour by this reagent<sup>4</sup>. Within 1 to 2 hours of extraction at room temperature, the nitrogen content of wheat flour was reduced from 2.31 to 0.03 per cent; most of the starch remained as an insoluble residue and less than 3 per cent of carbohydrates were solubilized in this procedure. The inclusion of urea in the extracting fluid was found to be of no special advantage.

It was of interest, therefore, to examine the effectiveness of this reagent in solubilizing the proteins of oilseed cakes which are apparently resistant to the peptizing action of alkali, such as sesame and mustard cakes<sup>2</sup>, which contrast sharply with peanut cake which gives an overall recovery of over 75 per cent nitrogen upon extraction with alkali in the pH range 7 to 8<sup>3</sup>. Since, to our knowledge, Swan's reagent has not been used in the extraction of proteins from plant materials other than wheat flour, a comparative study of the protein solubilizing powers of dilute alkali and Swan's reagent was made under identical conditions using a number of protein-bearing plant products.

## Experimental Materials and Methods

All the materials used in this study excepting *tinai*, *ragi* and *muringa* leaves were purchased locally. The *tinai* and *ragi* samples were obtained through the courtesy of the Director, Agricultural Research Institute, Coimbatore. The *muringa* leaves were collected from the Institute garden. The cereals, pulses and oil cakes were ground in a laboratory mill to yield a powder of 100 to 150 mesh size. The *muringa* and *palak* leaves, freed from stalks and stems were dried in the sun, subsequently at 70° in an air oven and then powdered. The pulverized materials were defatted with petrol ether (50 to 60° b.p.) in a Soxhlet extractor and used as such for protein extraction. 10 g. quantities of the defatted powders were dispersed in 200 ml of 0.2 per cent sodium hydroxide (pH of suspension was about 10.5) or in 200 ml of Swan's reagent which was prepared by mixing 5 ml of 1 M copper sulphate with 30 ml of 1 M ammonia, adding to it a solution of 1.575 g. of sodium sulphite in 50 ml water and diluting the resulting mixture to 250 ml. The extraction, washing and precipitation steps of the protein are outlined in the accompanying flow-diagram.





Results of this extraction procedure as applied to three cereals, two oil cakes, two pulses and two leaves are summarised in Table I as the Kjeldahl nitrogen values of the starting materials and the residues left behind after extraction of proteins.

TABLE I. Extraction of proteins from plant materials by alkali peptization and Swan's reagent

		Defatted powder	% N content of Alkali Residue	Swan's residue
Wheat	...	1.67	0.07	0.06
Tinai	...	1.85	1.67	1.31
Ragi	...	1.34	1.10	1.27
Arhar dal	...	3.42	1.00	0.98
Bengal gram dal	...	3.28	0.65	0.65
Mustard cake	...	7.16	4.45	4.57
Sesame cake	...	6.39	2.41	2.39
Muringa leaves	...	3.25	3.73	3.98
Palak leaves	...	4.18	5.80	7.19

Extraction of nitrogen by either of the methods gives good yields only with wheat, Bengal gram dal, arhar dal and to a lesser extent with sesame cake. About 30 per cent of the nitrogen is only extracted from mustard cake and very little nitrogen is solubilized from *tinai* and *ragi*. This is in conformity with earlier observations on the poor solubility of



*ragi* proteins in a variety of solvents<sup>5, 6</sup>. Presumably if *ragi* and *tinai* are processed by germination prior to extraction of proteins, the yields could be improved. The figures for nitrogen for the defatted leaf powders and their residues after extraction by alkali or Swan's reagent appear to indicate that the solvents employed remove preferentially some non-nitrogenous materials from the leaves and consequently enrich the residue in nitrogen as compared to the starting material. This effect is particularly striking in the case of *palak* leaves the nitrogen content of which increases nearly twice after extraction by Swan's reagent. In general, there appears to be no special advantage in Swan's reagent over the conventional alkali method in relation to recovery of nitrogen.

Protein yields and the nitrogen content of the products are given in Tables II and III.

TABLE II. *Yield of protein from defatted materials by extraction with alkali or Swan's reagent*

		Yield as % of starting materials	
		Alkali	Swan's reagent
Wheat flour	...	2.7	4.0
<i>Tinai</i>	...	1.0	1.5
<i>Ragi</i>	...	1.5	0.8
<i>Arhar</i> dal	...	16.2	17.7
Bengal gram dal	...	14.8	17.6
Mustard cake	...	22.6	25.4
Sesame cake	...	26.5	30.6

TABLE III. *Nitrogen content of proteins isolated by alkali or Swan's reagent*

		% Nitrogen		% Purity*	
		Alkali	Swan's reagent	Alkali	Swan's reagent
Wheat	...	12.30	12.00	77.5	75.0
Mustard cake	...	12.96	13.40	81.0	84.0
Sesame cake	...	12.60	12.60	79.0	79.0
<i>Arhar</i> dal	...	11.10	11.20	70.0	70.0
Bengal gram	...	13.00	12.40	81.0	77.5

\* On the basis of 16 % nitrogen in an ideal protein.



The figures for protein yields indicate somewhat slightly higher recovery using Swans' reagent than with alkali excepting in *ragi*. The recovery of nitrogen as protein from wheat appears to be rather low while comparing the figures for the nitrogen actually extracted and solubilized.

In order to find out whether time of contact of the materials with the extractants could affect the degree of solubilization of nitrogen extraction was followed for an eight hour period using 125 g. of defatted sesame cake and 2500 ml of the extractants. The results are given in Table IV.

TABLE IV. *Solubilization of nitrogen from sesame cake at different periods extraction*

Time of extraction (hours)		% Nitrogen in residue	
		Alkali	Swan's reagent
1	...	1.93	1.67
2	...	1.93	1.60
4	...	1.58	1.52
8	...	1.82	1.82

It would appear that the extraction is slightly better in four hours of contact than one to two hours and, after four hours, presumably resorption of nitrogen takes place.

The copper content<sup>7</sup> of the proteins prepared by extracting the above materials with Swan's reagent was found to be as high as 1 per cent. After prolonged dialysis against versene this could be brought down to 0.02 per cent. The susceptibility of the proteins isolated from alkali and Swan's extracts of sesame cake to digestion by papain was followed for six hour periods at 55°. In presence of versene, alcohol, precipitated papain brought about 20 per cent hydrolysis of the proteins indicating that the behaviour of the proteins in this respect is similar.

## Discussion and Conclusions

The results of the above study reveal no significant differences between alkali and Swan's reagent as solvents for the nitrogenous constituents of a number of plant materials. The yields of protein were slightly higher with the latter solvent. The proteins extracted by Swan's reagent were also more easily dispersible in water at neutral pH than the products obtained by alkali peptization. These advantages are, however, offset by the colour of the resulting protein. Extensive dialysis against versene removes most of the copper salts and thereby improves the colour of the protein; in practice, however, this method should prove to be uneconomical. The reagent has been reported to cause no structural changes in the protein excepting to open the disulphide bridges. The biological value of the protein isolated by this method is yet to be assessed. The extreme resistance of the millets *ragi* and *tinai* and the leaves of *muringa* and *palak* to the solubilizing action



of either alkali or Swan's reagent would suggest more fundamental studies on these materials to ascertain the mode of linkage of the proteins to other structural constituents like cellulose and hemicellulose.

### Acknowledgment

The author wishes to record his appreciation of the able technical assistance of Shri Vijay Verma in carrying out the above studies.

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### Discussion

- Q. Swan's reagent is known to cause degradative changes in proteins, as in the case of wool, where 80 per cent of the keratin is converted into the keratoses. What was the idea in comparing it with alkali for protein extraction?
- A. The above reagent is reported to degrade proteins by breaking up the disulphide bridges without causing any other changes. It has been used for the extraction of proteins from wheat, with satisfactory results. It was, therefore, of interest to study its efficiency for extracting protein from a number of vegetable sources. The results indicate that it is no better than alkali. There are also certain disadvantages attending the use of the reagent. We would like to warn against the use of the reagent as a general protein extractant.



# THE USE OF HEXAMETAPHOSPHATE IN THE ISOLATION OF BIOLOGICALLY ACTIVE BASIC PROTEINS

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This communication describes the results of a preliminary study of the complexing properties of sodium hexametaphosphate and their application in the isolation of basic proteins from tissue fluids.

Metaphosphoric acid has been employed as a deproteinizing agent in the estimation of various constituents of serum and cerebrospinal fluid<sup>1</sup>. Its most important use has, however, been in the assay of ascorbic acid in animal and plant tissues<sup>2</sup>, where, apart from precipitating out the proteins, hexametaphosphate protects the vitamin against auto-oxidation catalysed by heavy metals. Though a few patents have been granted for the isolation of proteins from milk whey and agricultural waste products using metaphosphoric acid<sup>3</sup>, its specific use as a precipitating agent for proteins has not been adequately explored.

## Experimental Results

Optimal conditions for the complex formation between sodium hexametaphosphate (Judex Chemical, General Chemical and Pharmaceutical Co., Sudbury, Middlesex, England, having an end group mol. wt. of 600) and a basic protein were worked out using crystalline egg white lysozyme (Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A.) as the standard. The combination was found to occur readily near a pH region of 4.0 in acetate or citrate-phosphate buffers, further shift in pH towards the neutral region causing dissolution of the precipitate. Approximately identical gram quantities of the two reactants combined under these conditions; for example, to completely precipitate lysozyme from a 0.05 per cent solution, the concentration of hexametaphosphate required was also near about 0.05 per cent. The complex formation was sensitive to changes in the ionic strength of the medium and, in presence of 1.0 per cent sodium chloride, no precipitation occurred. The experimental procedure routinely employed for following complex formation was to mix the freshly made solution of hexametaphosphate of appropriate strength with aqueous buffered extracts of the protein under investigation. On leaving the tubes undisturbed in an ice bath for about an hour, the complex settles as a heavy precipitate which can be readily separated from the medium by centrifugation.

The optimal conditions for the isolation of lysozyme from egg white by hexametaphosphate precipitation were standardized. A four fold dilution of fresh egg white was employed as the starting material. The most favourable concentration of hexametaphosphate was found to be in the range of 0.1 to 0.2 per cent. The precipitated enzyme could be readily eluted from the complex by 1 per cent sodium chloride in 0.005 M sodium acetate buffer, pH 4.0. The technique adopted finally as exemplified by the preparation of lysozyme from egg white is schematically represented below. The purification and yield of lysozyme by the above procedure are summarised in Table I.



Isolation of lysozyme from egg white by hexametaphosphate

4 times diluted egg white centrifuged at 5° for 30 min at 2000 r.p.m.

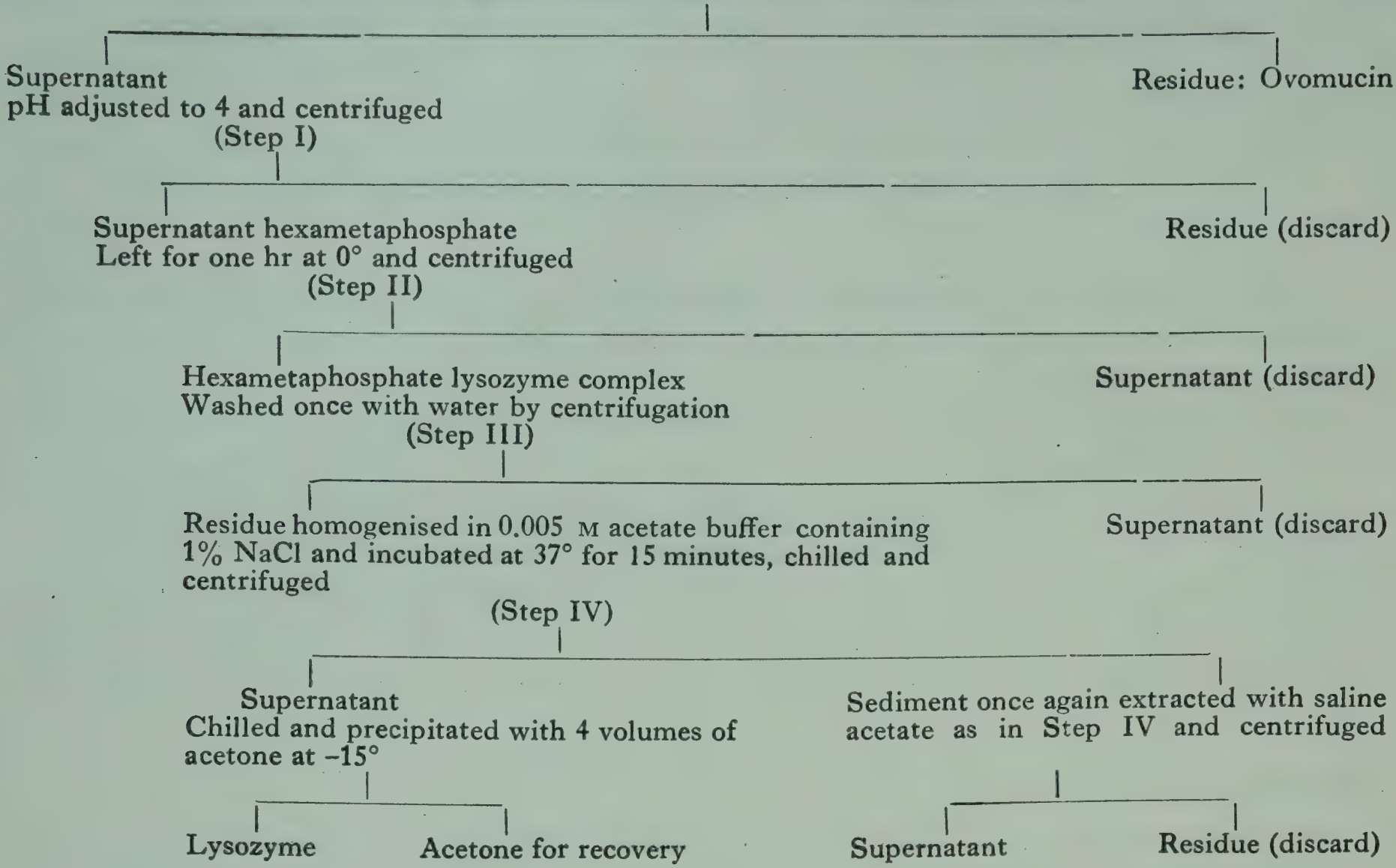


TABLE I. Preparation of lysozyme from egg white by precipitation with sodium hexametaphosphate

	Protein (mg/ml)	Activity units	Specific activity	Recovery %
Starting material (Egg white diluted four times)	20.8	600	28.8	
Step IV: elution with 1% sodium chloride ...	6.8	2200	323.0*	} 86.6
Second elution ...	1.5	380	260.3	

\* The specific activity will be much higher than this value, if it is corrected for the rather high Extinction coefficient value of lysozyme at 280 mμ as compared to bovine plasma albumin.

The above procedure enables the preparation of lysozyme with over ten-fold purification and a recovery of 86 per cent of the activity present in the starting material. It may be mentioned here that the conventional method of isolation of the enzyme from egg white involves adsorption on bentonite, elution by aqueous pyridine and dialysis followed by iso-electric precipitation of the amorphous material and crystallization.<sup>4</sup> Apart from



giving very poor yields, this method fails when applied to the separation of lytic enzymes from plant extracts (Shukla and Krishna Murti, unpublished observations).

*Preparation of lytic enzymes from rabbit spleen:* Lytic enzymes similar in properties to egg lysozyme are also present in rabbit spleen<sup>5</sup>. Under standardized conditions, these lytic enzymes could be directly precipitated from spleen extracts by the use of hexametaphosphate. In a typical experiment, 0.25 M sucrose homogenates of a rabbit spleen were allowed to freeze and thaw and centrifuged for thirty minutes at 10,000 r.p.m. in rotor No. 30 of the Spinco Ultracentrifuge Model L. The clear supernatant was employed as the starting material for the isolation of lytic enzymes. Aliquots of this were mixed with 0.1 M acetate buffer pH 4 containing varying concentrations of hexametaphosphate in the range 0.001 to 1.0 per cent. The tubes were kept in an ice bath for one hour and centrifuged at 2000 r.p.m. at 5° for fifteen minutes. Protein and lytic activity were determined in the supernatants. Protein was estimated spectrophotometrically<sup>6</sup> and lytic activity by following the decrease in turbidity of suspensions of *Micrococcus lysodeikticus* NCTC—2665.<sup>7</sup> The results are represented in Fig. 1. It is clear that the lytic activity of the spleen like the lysozyme of egg white could be readily precipitated by hexametaphosphate at pH 4. Protein and activity run almost parallel in the precipitation scheme, although, in the range of 0.025 to 0.5 per cent of hexametaphosphate, all the activity is precipitated whereas 20 per cent of the proteins are still in the supernatant.

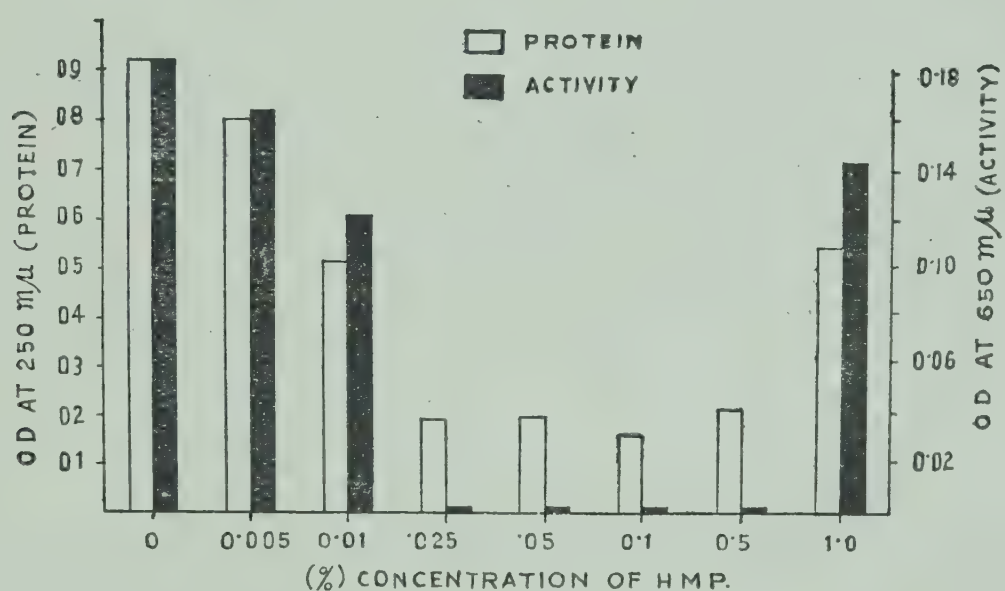


FIG. 1: Precipitation of Rabbit Spleen lysozyme by hexametaphosphate.

*Preparation of basic proteins from rabbit liver nuclei:* Rabbit liver nuclei were separated from 0.25 M sucrose homogenates of the tissue adopting the procedure of Hogeboom and Schneider<sup>8</sup>. The nuclei sedimented at 800 g. were extracted with 0.25 N hydrochloric acid and the extracts frozen and thawed thrice successively to release the histones. The extracts were then centrifuged and the supernatants which carried most of the nuclear histones were dialysed in running water till the pH of the dialysate corresponded to that of water. The precipitate that separated out in the dialysis bag was centrifuged off, dissolved in 0.1 M acetate buffer and used in precipitation studies with hexametaphosphate. In an effective concentration of 0.01 per cent, practically all the proteins were precipitated. In striking contrast, however, to the behaviour of the lytic enzymes of egg white and rabbit spleen, the complex of liver nuclei histone and hexametaphosphate could not be dissociated even by 20 per cent hexametaphosphate or sodium chloride. Addition of Ca and Mg ions up to 0.1 M also did not help in the dissociation.



*Purification of the lytic enzyme of the latex of Calatropis procera:* The latex possesses a very powerful lytic activity against gram-positive bacteria (Shukla and Krishna Murti, unpublished observations) and all attempts so far to isolate the enzyme from the latex in a high degree of purity by the bentonite adsorption procedure have been futile. Exploratory trials have, however, indicated that hexametaphosphate in specified concentrations removes a major part of the interfering proteins leaving the enzyme in solution. As a starting material for purification of the enzyme, the hexametaphosphate supernatant would appear to be more suitable than the latex as such.

## Conclusions

The results of these preliminary investigations would thus indicate the usefulness of hexametaphosphate as a precipitating agent in the isolation of biologically active basic proteins. If favourable conditions are worked out, it is possible either to precipitate directly the protein under investigation or to remove inert proteins from the source of the active material. The results presented also suggest that investigations on trypsin inhibitors, polypeptide hormones and antibiotics might be facilitated by the complexing property of hexametaphosphate if judiciously employed under standardized conditions.

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## Discussion

Q. What was the purity of the hexametaphosphate? was the molecular complexity ascertained?

A. The reagent used was of analytical grade. Tests carried out by Prof. P. S. Krishnan at the Department of Biochemistry, Lucknow University, showed that it was homogeneous.

Q. How does the method compare with the standard procedures?

A. A number of difficulties are encountered with the standard procedures for isolating and purifying lysozyme. The procedures are not only involved but the yields are poor. Some of the procedures also fail when applied to plant sources. Our results illustrate the superiority of hexametaphosphate in the isolation of lysozyme and other basic proteins. Since only 0.1–0.2 per cent concentrations are employed it would be more economical than ammonium sulphate fractionation procedures. It may also prove a very useful reagent for the isolation of trypsin inhibitors.



# ESTIMATION OF IODINE IN PROTEIN-IODINE COMPLEXES

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Determination of iodine in the presence of certain ions is often difficult, specially in the presence of halides and when a polyvalent ion is present in the solution. In presence of proteins the end-point is difficult to determine.

During studies on a protein-iodine complex it was necessary to determine the iodine in very small amounts in the presence of excess of proteins. The usual method in such cases is to add excess of silver nitrate to the solution in the presence of nitric acid and back-titrate the silver nitrate with ammonium thiocyanate using ferric alum as the indicator. In the presence of excess of protein, determination of the end point becomes difficult. The conversion of the iodide into iodate is a lengthy and complicated process.

An attempt was, therefore, made to separate the iodide ions from the protein or peptides by a simple method and then titrate directly. The anion-exchange resin 'Decolorite' was found to be suitable for adsorption of the iodide ions followed by subsequent elution. A simple method, the details could then be worked out for the determination of iodine, of which are given below.

## Experimental Materials

1. 'Decolorite', an anion-exchange resin (Permutit & Co. Ltd., U.K.).
2. Sodium hydroxide, Analar (B.D.H.). A semi-normal solution of this was prepared in distilled water.
3. Acid Formic, 98-100 per cent crystallisable (E. Merck). A 1 per cent solution of this in distilled water was used.
4. Acid Acetic Glacial G. R. (E. Merck).
5. Silver nitrate A. R. (Johnson), 0.01 and 0.002 N solutions were prepared from this.

## Method of analysis

*Preparation of the column:* A chromatography column 10 cm in length and 1 cm in diameter was prepared with 5 g of the resin. It was washed with water and 1 per cent solution of formic acid was passed through it when the pH of the effluent was less than 2.0. It was then washed with distilled water till the pH of the effluent was 4.0.

An aliquot of the protein-iodine complex solution was now percolated through the column followed by a few ml of distilled water to remove the protein completely. The percolate was found to contain protein and peptides free from iodine. The iodide adsorbed on the resin was eluted by passing two to three bed volumes of the sodium hydroxide



solution depending on the amount of iodide present. A few ml of distilled water was passed through the column to elute the iodide completely. The solution was neutralised with acetic acid and titrated against silver nitrate using eosin as the indicator.

## Results

In Table I are given the results of analysis of iodide solutions with and without protein.

TABLE I

No.	Protein present or absent	Iodide	
		Found (mg)	Present (mg)
1.	Absent	14.86	15.0
2.	„	7.18	7.2
3.	Present	14.11	14.2
4.	„	13.87	14.0
5.	„	5.04	5.0
6.	„	6.93	7.0
7.	„	2.52	2.5
8.	„	0.255	0.252
9.	„	0.130	0.129

## Discussion

A simple method was thus evolved by which iodide could be determined in the presence of a large excess of protein or peptides.

In the initial stages of the work, nitric acid A. R. was used for the neutralisation of the alkali, but the end-point was not sharp and iodide less than 1 mg was difficult to determine. In quantities less than 100 g the end point could not be detected.

The use of acetic acid greatly enhances the sensitivity of the indicator and, by careful adjustment of the volume of the titrant and the concentration of the titrating solution, iodide upto about 10 microgram could be determined. Use of starch-iodide as the indicator seems to be superior to this and work is in progress on this aspect.

## Acknowledgment

The authors wish to thank Dr U. P. Basu for his interest in the work.

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# STUDIES ON 'IODOTHYRINS'

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Despite the considerable advances made in our knowledge of the biochemistry and physiology of the thyroid hormone, little is known regarding the chemistry of the macromolecular storage matrix of the hormone namely, thyroglobulin. Thyroglobulin prepared by the method of Roche *et al*<sup>1</sup>, has a molecular weight of 6,50,000 and is a glycoprotein<sup>2,3</sup>. N—terminal amino acid analysis of thyroglobulin has revealed that as many as 10-amino acids are present at the amino end of the protein and, on this basis, a multi-branched structure has been assigned to the protein<sup>4</sup>.

Many attempts have been made in the past to isolate from partial acid and enzymic digests of thyroglobulin, crystalline iodine-containing compounds possessing high physiological activity. Since the isolation of thyroxine, however, these ill-defined compounds, termed 'iodothyryns', lost much of their importance.

The present study covers a detailed investigation on the nature of 'iodothyryns' employing several recent techniques of protein analysis. From a partial acid hydrolysate of cattle thyroglobulin, two iodopeptides have been isolated and characterized. The properties of similar fragments from sheep and hog thyroglobulins have also been investigated.

## Experimental Materials

*Thyroglobulin*: Cattle and sheep thyroglobulins were prepared by the method of Roche *et al*<sup>1</sup>. Hog thyroglobulin, prepared by acidification of a saline extract of the thyroid glands, was a gift from Dr R. L. Kroc of Warner-Chilcott Research Laboratories, New York, N. J.

*Dinitrophenyl (DNP-) amino acids* were commercial preparations obtained from the Mann Research Biochemicals, U.S.A.

*Buffers for electrophoresis*: Pyridine-acetic acid buffers (pH 5.0 and 4.2) were prepared by the method of Wilcox *et al*<sup>5</sup>. The other buffers used in these studies were prepared according to standard tables<sup>6</sup>.

## Methods

*Paper electrophoresis* was carried out in the horizontal open type apparatus (Arthur Thomas Co., Philadelphia, U.S.A.) using Whatman No. I filter paper strips (4 × 30 cms) conducted usually at 250 volts.

*Moving boundary electrophoresis* of the peptides was carried out through the courtesy of Dr V. Jagannathan, National Chemical Laboratory, Poona.

Nitrogen in the protein and peptide samples was estimated by the colorimetric Nessler procedure, as modified by Koch and McMeekin<sup>7</sup>.

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Iodine was estimated by the procedure of Kendall as modified by Harington<sup>8</sup>.

Hexosamine analysis was carried out by the modification of the Elson-Morgan procedure, detailed by Winzler<sup>9</sup>. A 6 hour hydrolysis period was employed and glucosamine hydrochloride was used as the standard. Amino acid analysis of the various fractions was carried out by the procedure of Giri *et al.*<sup>10</sup>, after separation of the amino acids by two dimensional paper chromatography.

N-terminal amino acid analysis of the peptide fractions was carried out essentially by the procedure outlined by Levy<sup>11</sup>. Paper chromatography of DNP—amino acids was carried out in the tert—amyl alcohol-phthalate (pH 6.0) system and or in 1.5 M phosphate buffer (pH 7.0)<sup>11</sup>. Water soluble DNP-amino acids were chromatographed in n-butanol-acetic acid-water (4:1:5) system.

## Results

The homogeneity of the protein samples was checked by electrophoresis on paper and agar before the degradation of the proteins into smaller peptide fractions was attempted.

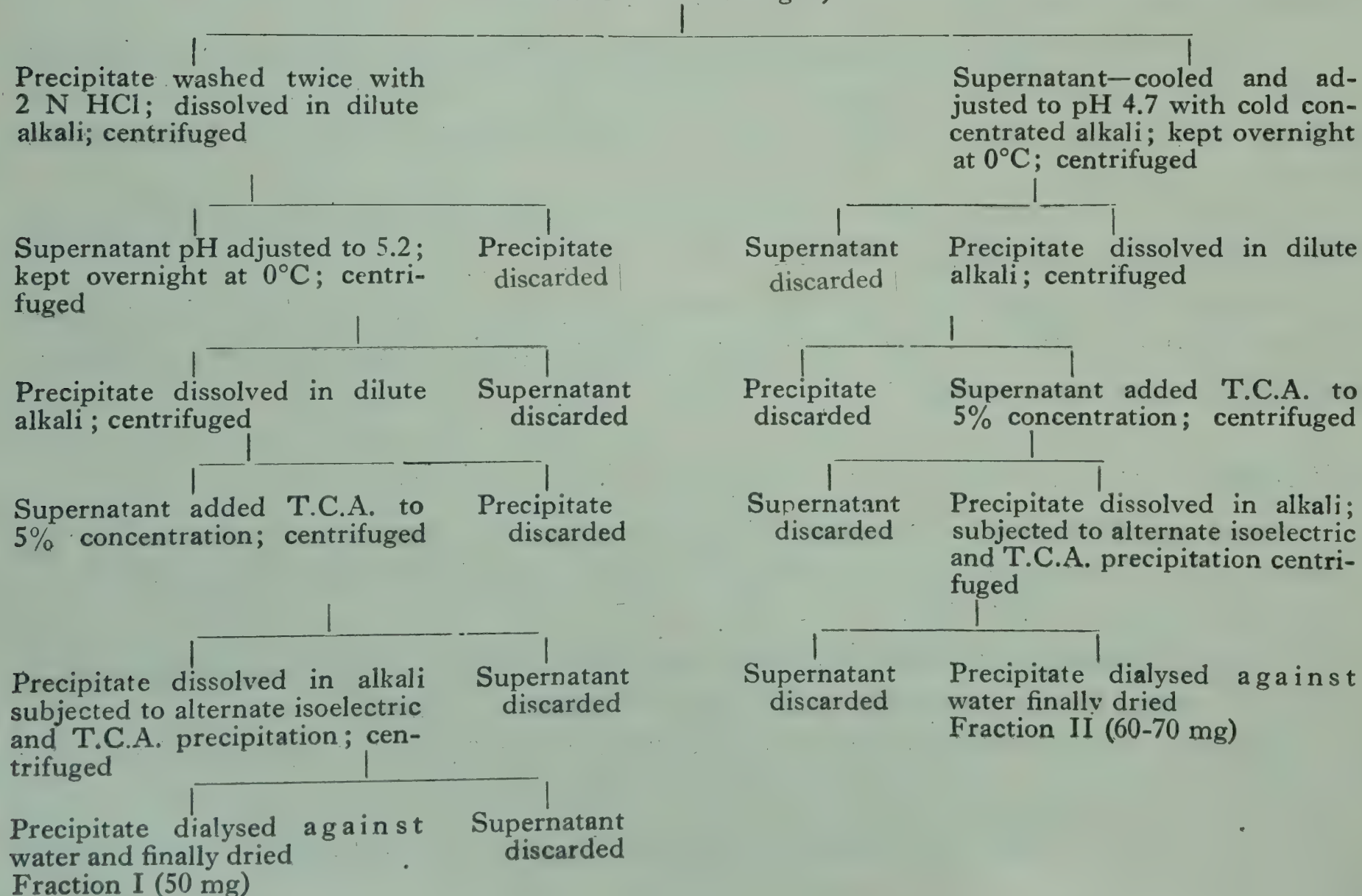
*Partial acid hydrolysis of cattle thyroglobulin and isolation of peptide fractions:* Preliminary experiments showed that partial hydrolysis with conc. HCl was a convenient method for the degradation of the protein.

The procedure finally adopted for the isolation of iodopeptides is shown in the accompanying scheme.

### *Scheme for the isolation of iodopeptides from partial acid hydrolysate of cattle thyroglobulin*

#### Cattle thyroglobulin

(1 g in 50 ml of 10 N HCl incubated at 37°C for 6 hours; diluted to 250 ml: kept overnight at 0°C and centrifuged)





The six hour incubation period was chosen after several experiments employing various periods of hydrolysis. While hydrolysis for 16 hours yielded material corresponding to Fraction I which had a higher iodine content, the yield of the product was low. Incubation periods of less than 6 hours led to a complex mixture of several peptides and also frequently contained undigested thyroglobulin. The usefulness of each step in the isolation scheme in the purification of the peptides was followed by paper electrophoresis.

*Characterization of Fractions I and II:* The methods of isolation and purification of Fractions I and II reveal a few of the properties of these peptides. Thus both are TCA insoluble and non-dialyzable. The isoelectric points of Fractions I and II are 5.2 and 4.7 respectively. The nitrogen and iodine contents of the two peptides as also of the parent proteins are given in Table I.

The homogeneity of Fraction I was examined by electrophoresis in several buffers of pH ranging from 4.0 to 9.0. Several reagents specific for the different groups in the protein were used for locating the compound. The positions of the bands as revealed by spraying with the various reagents were identical. The homogeneity of the fraction was also confirmed by moving boundary electrophoresis experiments, wherein only one peak was seen. Similar results were obtained with Fraction II.

*Hexosamine in the peptides:* Since it is now well established that thyroglobulin is a glycoprotein, it was of interest to study whether the fractions retained the mucoid characteristics of the protein. The peptides were analysed for their hexosamine contents and the values are recorded in Table I. Both the fractions contain varying amounts of hexosamine. The hexosamine contents of cattle and sheep thyroglobulins are of the same order as reported previously for the protein from other species<sup>2</sup>.

TABLE I. Nitrogen, iodine and hexosamine contents of thyroglobulin, Fraction I and Fraction II of different species

Species	Compound	Nitrogen %	Iodine %	N : I atomic ratio	Hexosamine %
Cattle	Thyroglobulin	15.5	1.08	130.2	2.60
	Fraction I	13.5	4.13	29.7	0.48
	Fraction II	12.5	2.19	51.8	0.95
Sheep	Thyroglobulin	15.0	1.05	139.6	1.20
	Fraction I	14.5	3.62	36.3	0.42
	Fraction II	13.0	2.26	52.2	0.81
Hog	Thyroglobulin	15.9	1.07	134.8	1.70
	Fraction I	14.0	4.12	30.8	0.56
	Fraction II	12.2	1.83	60.5	0.86

*Amino acid analysis of cattle thyroglobulin and Fractions I and II:* The results of the analysis on duplicate samples of the iodopeptides from cattle thyroglobulin are presented in Table II.

*End group analysis of Fractions I and II:* The constituent N-terminal amino acids of Fractions I and II, were analysed by the FDNB-technique of Sanger. Examination of ether soluble DNP-amino acids showed the presence of DNP derivatives of aspartic



TABLE II. *Amino acid composition of Cattle thyroglobulin and Fractions I and II*  
(Values expressed as g. amino acid per 100g. protein/peptide)

Amino acid	Thyroglobulin*	Fraction I*	Fraction II*
Alanine	7.92	4.10	4.40
Arginine	11.89	13.38	10.80
Aspartic acid	6.88	6.81	3.41
Cystine	3.36	5.14	3.38
Glutamic acid	12.50	13.30	10.51
Glycine	4.40	2.50	2.57
Lysine	3.47	2.77	2.34
Leucine/Isoleucine	13.30	15.00	14.80
Methionine, valine	6.52	7.05	7.51
Phenylalanine	6.34	6.34	6.50
Proline	8.90	7.90	...
Serine	3.39	2.70	2.21
Threonine	3.10	1.71	1.92
Tyrosine	2.83	4.10	3.94
Thyroxine	0.38	1.29	1.10

\* Values uncorrected for moisture.

acid, alanine and serine in Fraction I and the same were present in hydrolysates of DNP Fraction II. In addition, small amounts of DNP-glutamic acid were observed in Fraction II.

*Comparative studies on Fractions I and II obtained from thyroglobulins of different species:* A comparative study of the properties of the peptide fractions isolated from hog, sheep and cattle thyroglobulins was carried out.

The peptides were isolated from the partial acid hydrolysates by the method indicated earlier. In Table I are given the nitrogen, iodine and hexosamine values for the different fractions. These data point out the essential similarity of the various fractions.

Data on paper electrophoresis studies of the fractions obtained from the thyroglobulins of different species showed that the corresponding fractions had identical mobilities. Moving boundary analysis of Fractions I and II isolated from hog thyroglobulin also established their homogeneity.

The amino acid contents of Fractions I and II prepared from hog and sheep thyroglobulins were estimated and the values are presented in Table III.

End group analyses showed that aspartic acid, alanine and serine are present at the N-terminus of Fraction I obtained from the proteins of the three species while aspartic acid, glutamic acid, alanine and serine occupy the amino end of Fraction II.

## Discussion

The importance of working with large peptides in structural studies on proteins as emphasized by Sanger, becomes all the greater in the case of high molecular weight proteins. The present investigation demonstrates the suitability of partial acid hydrolysis for the preparation of iodopeptides from thyroglobulin. The two peptides isolated by this method are electrophoretically homogeneous. They bear striking resemblance to thyroglobulin in their amino acid composition and in having  $P_i$  very close to that of the latter.



TABLE III. *Amino acid composition of Fraction I and Fraction II isolated from hog and sheep thyroglobulins*

(Values expressed as g. amino acid per 100 g. peptide)

Amino acid	Hog		Sheep	
	Fraction I*	Fraction II*	Fraction I*	Fraction II*
Alanine	5.0	7.27	4.80	8.10
Arginine	10.20	9.45	11.10	11.40
Aspartic acid	6.95	5.46	6.10	5.77
Cystine	3.52	3.50	8.10	2.71
Glutamic acid	10.31	10.40	11.31	10.14
Glycine	2.21	2.52	2.32	2.93
Lysine	2.52	3.10	3.61	2.50
Leucine/Isoleucine	14.91	15.76	20.0	16.09
Methionine, valine	6.06	5.63	8.40	7.21
Phenylalanine	7.89	6.26	3.53	6.26
Proline	6.29	...	7.00	...
Serine	2.96	2.82	3.30	2.83
Threonine	2.40	3.10	2.52	8.10
Thyroxine	1.27	1.01	1.23	1.05
Tyrosine	3.10	3.30	1.98	3.94

\* Values uncorrected for moisture.

Further, they still retain the mucoid characteristic of the original protein. The close similarity of the thyroglobulin preparations from various species is reflected also in the properties of the peptide fractions isolated from them. Fractions I obtained from hog, cattle and sheep thyroglobulins have identical electrophoretic mobilities and show preponderance of the same amino acids. The same remarks apply equally well to the Fraction II of the different species. Further, Fractions I obtained from all the three proteins have higher iodine and lower hexosamine contents than the corresponding Fraction II. The most notable point of resemblance, however, is that the respective N-terminal amino acids of Fractions I and II are the same in all the three cases.

It is interesting to note that these fractions resemble the acid-insoluble, partial degradation products—'iodothyryns' of the earlier workers<sup>13,14</sup>—especially in their N: iodine ratios. In the light of the present investigations, these iodothyryns which are homogeneous peptides and certainly are far less complex than the parent protein, assume immense significance and are not merely of historical interest. The homogeneity of the fractions, their high thyroxine content and the relatively low hexosamine content indicate that the isolation of these peptides is an important and useful step towards establishing the structure of thyroglobulin.

### Acknowledgment

The authors wish to acknowledge the active collaboration of Dr N. R. Moudgal in the earlier phases of this work. The major part of this work was carried out in the University Biochemistry Department, Madras, under a research scheme, financed by the Council of Scientific and Industrial Research, New Delhi.



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*Discussion*

- Q. How do the Fractions I and II compare in respect of thyroidal activity?
- A. Fraction I is more active than Fraction II.
- Q. Does the carbohydrate moiety affect the activity?
- A. It is not known whether the carbohydrate component influences the activity. Presumably the structure of the peptide mainly determines the activity.
- Q. Could the differences in activity be due to loss of any component?
- A. It is difficult to say since the peptides were isolated from partial hydrolysates of the parent thyroglobulin.



# A SIMPLE PROCEDURE FOR SEPARATING PROTEINS IN THE ISOLATION OF PENTOSE-NUCLEIC ACID AND DESOXPENTOSE-NUCLEIC ACID FROM MAMMALIAN TISSUES

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In any investigation on the structure of nucleic acids the mode of isolation should be specified. The conflicting reports which have arisen may be due to the fact that the authors have compared nucleic acids isolated by different methods. The majority of the early methods of isolation were drastic enough to produce depolymerisation and possibly subsequent repolymerisation of the macro-molecules. Dilute alkali has often been used to extract the nucleic acid and hydrochloric acid or acetic acid for precipitation of the extracted material. Later work has been directed towards the development of mild methods of isolation in order to minimise the degradation. Most of these methods involve isolation of nucleoprotein and subsequent deproteinisation. One of the earlier mild methods of isolation of nucleoprotein from the thymus was that due to Hammersten<sup>1,2</sup> and Bang<sup>3</sup> who used water to extract the nucleoprotein and calcium chloride for its precipitation. The most useful method for the isolation of desoxyribonucleic acid (DNA) is that of Mirsky and Pollister<sup>4</sup> in which the nucleoprotein is extracted with 1 M sodium chloride and re-precipitated by dilution of the solution to 0.14 M.

The classical method of removing proteins from nucleoprotein is the shaking of the solution of the nucleoprotein in 1.0 M sodium chloride with a mixture of chloroform and octyl alcohol (or amyl alcohol)<sup>5, 6</sup> when the denatured protein could be separated out as a gel by centrifugation. This method is tedious and the gel thus formed causes much loss of ribonucleic acid (RNA) through adsorption<sup>7</sup>. Volko<sup>8</sup> and Putman<sup>9</sup> were the first to indicate that protein could be precipitated by surface active agents and subsequently Marko and Butler<sup>10</sup>, Bernstein<sup>11</sup>, Jones<sup>12</sup> and Dutta *et al*<sup>13</sup>. reported the application of surface active agents. Grinman and Mosher<sup>14</sup> reported the use of guanine hydrochloride for the denaturation of protein. Lately, Kirby<sup>15</sup> has used phenol for the removal of protein from ribonucleoprotein. All the above methods are suitable for isolating either DNA or RNA alone. Simultaneous isolation of both the nucleic acids from mammalian tissues was a difficult problem, about which the present work is concerned.

## Experimental

Calf liver (25 g) was homogenised with urea solution (75 g urea dissolved in 125 ml of distilled water) and stirred for 6-8 hours. Sodium chloride (7.5 g) was added to it and stirring continued for another 2 hours. The mass was then centrifuged (4000 r.p.m./20 min.) to remove the insoluble cell debris. The supernatant solution was precipitated with ethanol (2 vol.) and the harvested precipitate was thoroughly triturated with 8 per cent sodium chloride solution (50 ml). The insoluble residue was discarded by centrifugation and the clear solution was finally clarified by shaking with a mixture of chloroform and



octyl alcohol once or twice. From this clear aqueous solution the nucleic acid was isolated and fractionated thus. The solution was freed from salts by simple dialysis against distilled water and then treated with *cetrimide* to precipitate down the nucleic acids. The complex precipitate was then suspended in 0.5 M sodium chloride (25 ml) and stirred thoroughly for some time, when the RNA went into solution leaving behind insoluble DNA. The RNA was precipitated with ethanol (2 vol.), washed with ethanol and ether and dried in vacuum over phosphorus pentoxide, the yield being 45-50 mg. The DNA residue was then triturated with 8 per cent sodium chloride (25 ml) very thoroughly and centrifuged, when a part of the DNA went into solution, and the other part remained insoluble. The soluble fraction of DNA (Fraction I) was subsequently precipitated with ethanol and washed and dried as above (DNA—I), the yield being about 20 mg. The insoluble DNA (Fraction II) residue was thoroughly triturated with ethanol to remove the *cetrimide* as far as possible. The insoluble nucleic acid was then dissolved in distilled water (15 ml) and then precipitated with ethanol (30 ml) in presence of sodium chloride (1 M concentration). The DNA—II thus obtained was washed and dried as above, the yield being about 18 mg. These nucleic acids, thus isolated, were analysed as shown in Table I.

TABLE I

Nucleic Acid	N%	P%	N/P	RNA %	DNA %	E <sub>258</sub> m $\mu$ (60 $\mu$ g/ml)	E <sub>260</sub> m $\mu$ (60 $\mu$ g/ml)	E <sub>max</sub> /E <sub>min</sub>
RNA ...	14.93	8.7	1.71	95	3.4	1.27	...	2.21
DNA—I ...	13.82	9.05	1.52	2.3	98.0	...	1.15	2.39
DNA—II ...	13.78	8.7	1.58	1.0	99.0	...	1.16	2.32

E = optical density.

Ultraviolet absorption analysis of the aqueous solution of these fractions of nucleic acids (60 $\mu$ g per ml) have shown that the RNA has a sharp maximum absorption at 258m $\mu$  and minimum at 228 m $\mu$ , while DNA fractions have maximum at 259-260 m $\mu$  and minimum at 230 m $\mu$ .

## Discussion

The isolation of nucleic acid from any particular source is a major problem to be solved before any study of the properties and structure of these high polymers can be undertaken. The method should be such that both RNA and DNA could be isolated as completely as possible under mild conditions.

The solution of the medium used for extracting the nucleoprotein from the cellular material is to be kept at pH value round about 7.0 and there is probably no particular advantage in using the usual sodium chloride in the extracting medium, since the nucleoprotein of the calf liver seems to be readily soluble at low ionic strength. Urea has got a very low dissociation constant and has a solubilizing property too.

No definite knowledge has yet been acquired with regard to the problem of inhibiting the ribonuclease and desoxyribonuclease in the tissue during isolation. Lately



Resnick and Carter<sup>16</sup> have reported that urea may deactivate nucleases by disrupting the disulphide bridges. Our experimental observation also seems to corroborate this hypothesis.

Removal of protein from nucleoprotein is a complex job. Several treatments with mixtures of chloroform-octyl alcohol<sup>5</sup> often cause distinct losses of RNA through adsorption. So in the present work the process was so adjusted that almost all proteins were removed by denaturation, only the last traces being removed by one or two treatments with chloroform-octyl alcohol mixture.

It is now well established that the internucleotide H-bonds in DNA and RNA may often be broken up during isolation. The method using surface active agents breaks up the H-bonds, often irreversibly, while the phenol method preserves much of the native structure of RNA. The ultra-violet absorption analysis of RNA in presence of urea shows that RNA does not have tightly paired chains bound by H-bonds like DNA, but consists of a disordered array of twisted chains with irregular location of H-bridges. Transfer of the RNA from urea solution back to phosphate buffer solution gives a complete reversion of the UV-absorption. However, the effect of urea on the structure of nucleic acids, particularly RNA, should be studied more thoroughly to evaluate the above method of isolation.

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# VITAMIN B<sub>12</sub> AND PROTEIN BIOSYNTHESIS

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The role of vitamin B<sub>12</sub> in protein metabolism was suggested almost at the beginning of the work on this vitamin<sup>1, 2</sup>. A lowering of the transmethyrase activity in the deficient rat liver was observed by Oginsky<sup>3</sup> and by William and co-workers<sup>4</sup>, which was confirmed by Mistry *et al.*<sup>5</sup>, for the rat, although not for either the pig or the chick. A lowering of other enzymes in vitamin B<sub>12</sub> deficiency was observed by Murthy *et al.*<sup>6</sup> and Wong and Schweigert<sup>7</sup>.

Mulgaonkar and Sreenivasan<sup>8</sup> reported a lowered serum protein level in vitamin B<sub>12</sub> deficient rats. Other evidences of a derangement of protein metabolism, are the reports of Schultze *et al.*<sup>9</sup>, of high blood urea in deficient rats and that of Charkey *et al.*<sup>10</sup>, of increase in blood free amino acids in vitamin B<sub>12</sub> deficient chicks. Vannoti<sup>11</sup> has also suggested that, in protein metabolism, vitamin B<sub>12</sub> is directly or indirectly concerned with protein biosynthesis.

While these indirect evidences were available, Wagle and Johnson<sup>12</sup> reported a lowering of amino acid incorporation into protein of deficient liver homogenates. A series of reports from this laboratory<sup>13-17</sup> indicated that vitamin B<sub>12</sub> is present in a higher concentration in 'pH 5-enzymes' as compared to the other sub-cellular fractions. Moreover we found that vitamin B<sub>12</sub> was concentrated in the 20-60 per cent ammonium sulfate fraction (ASF) of pH 5-enzymes (ASF) which was also the active portion of the pH 5-enzymes.

As the activating enzymes of practically most of the amino acids have been shown to be present in the 40-60 per cent ASF of pH 5-enzymes, we suggested that, perhaps, vitamin B<sub>12</sub> was involved in the activation of amino acids and probably formed a part of the activating enzyme.

Some time after we hypothesised the function of vitamin B<sub>12</sub>, Fraser and Holdsworth<sup>18</sup> reported a negative effect of B<sub>12</sub> in protein biosynthesis. They found that there was very little vitamin B<sub>12</sub> present in pH 5-enzymes of the chick, contrary to the amount found by us<sup>14</sup> in similar studies with rats. At the same time, Arnstein and Simkin<sup>19</sup> reported a lower incorporation of the amino acids into protein *in vivo* in deficient rats. Addition of vitamin B<sub>12</sub> *in vitro* did not increase amino acid incorporation in their studies. Dinning and Young<sup>20</sup> obtained similar results using chick bone marrow cells. This controversy inspired us to further follow up this line of work.

## Experimental Materials and Methods

For details see references 13-17 and 22.

## Results and Discussion

Using deficient and normal chick systems, we studied the incorporation of the amino acid into protein. However, in some of the incubation mixtures we added along with

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microsomes and pH 5-enzymes, the supernatant after the removal of pH 5-enzymes (SUP). The results of this experiment are summarised in Table I.

It is quite evident from the data in Table I, that SUP is required for the incorporation of amino acid into protein in the case of chick liver preparation.

TABLE I. Incorporation of  $C^{14}$   $H_3$ -methionine into normal and vitamin  $B_{12}$ -deficient systems of chicks

		Nutritional status of Chicks	
		$B_{12}$ -deficient, cpm./mg. protein	$B_{12}$ -normal cpm./mg. protein
Complete system (C.S.)	...	169.28	173.81
C.S. + Sup.	...	169.47	369.51
C.S. + $B_{12}$	...	177.62	175.69
C.S. + $B_{12}$ + sup.	...	248.82	360.86
C.S. + anti- $B_{12}$	...	135.94	109.18
C.S. + mixture of amino acids	...	242.82	311.5
C.S. + mixture of amino acids + sup.	...	271.97	430.5

Sup.=supernatant after the removal of pH 5-enzyme. The complete system contained 0.3 ml. of microsomes (7.5 mg. protein), 0.2 ml. of pH 5-enzymes (2.0 mg. protein), 0.25  $\mu$ mole of  $C^{14}$   $H_3$ -methionine, 10  $\mu$ moles PEP, 0.02 mg. PK, 0.5  $\mu$ mole ATP, and 0.025  $\mu$ mole GTP. Final volume made to 1 ml. by addition of 0.15 M KCl.

Abbreviations: ATP—Adenosine triphosphate; GTP—Guanosine triphosphate;  
PEP—Phospho enol pyruvate; PK—Pyruvic kinase.

Distribution studies of vitamin  $B_{12}$  in sub-cellular fractions were carried out by using  $Co^{60}$  labelled vitamin  $B_{12}$ , the results of which are presented in Table II.

It is obvious that SUP has maximum concentration of vitamin  $B_{12}$ , but at the same time vitamin  $B_{12}$  could not stimulate the incorporation of amino acid in the absence of SUP.

TABLE II. Distribution of radioactive vitamin  $B_{12}$  into liver subcellular fractions of chick

		Total cpm.	% activity in each fraction
Whole homogenate	...	176,000	100
Nuclei, unbroken cells	...	48,125	27.3
Mitochondria	...	3,275	1.86
Microsomes	...	10,080	5.7
pH 5 enzymes	...	3,960	2.25
pH 4.5 enzymes	...	13,320	7.57
Supernatant	...	76,100	43.24

The results in Tables I and II also explain the failure of Fraser and Holdsworth in indicating the role of vitamin  $B_{12}$  in amino acid incorporation as *in vitro* they had used only pH 5 enzymes and microsomes. From our results, it can be seen that addition of SUP to the system boosts up the incorporation even in the normal preparation. Thus it seems, that



in chick liver preparation, SUP is needed for the amino acid incorporation. Similar results have been obtained by Grossi and Moldave<sup>21</sup> in the case of rats.

The action of various inhibitors of vitamin B<sub>12</sub> and its reversal by the vitamin was also studied<sup>22</sup> and it was noted that vitamin B<sub>12</sub> failed to reverse the inhibition by these antagonists. CN is known to inactivate vitamin B<sub>12</sub> as the vitamin along with CN forms a di-cyano compound which does not possess the activity of the vitamin; and so we thought of studying the inhibition of amino acid incorporation by CN and the effect of addition of vitamin B<sub>12</sub> on the same. The results obtained are given in Table III.

TABLE III. *The inhibitory effect of KCN on the incorporation of 3-C<sup>14</sup>-phenylalanine and the effect of crystalline vitamin B<sub>12</sub> on the reversal of the inhibition*

		Supplement to the incubation mixtures	
		None, cpm./mg. protein	250 mγ crystalline B <sub>12</sub> cpm./mg. protein
Complete system (C.S.)	...	139.5	
C.S. + 10 <sup>-3</sup> M KCN	...	9.5	63
C.S. + 10 <sup>-4</sup> M KCN	...	20.0	66
C.S. + 10 <sup>-5</sup> M KCN	...	22.0	66
C.S. + 10 <sup>-6</sup> M KCN	...	55.5	86
C.S. + 5 × 10 <sup>-6</sup> M KCN	...	57.5	92.0

The complete system contained 0.3 ml microsomes (7.5 mg protein) 0.2 ml pH 5 enzymes (2.0 mg protein), 10 μmoles PEP, 0.02 mg PK, 0.25 μmole 3-C<sup>14</sup>-phenylalanine, 0.5 μmole ATP, 0.25 μmole GTP. Final volume made to 1.0 ml by addition of 0.15 M KCl.

It can be clearly seen that vitamin B<sub>12</sub> considerably reverses this inhibition of CN and stresses thus its role in amino acid incorporation.

The deficient microsome preparation did not affect the incorporation of the amino acid into protein, and at the same time deficient pH 5-enzymes lowered the incorporation of the amino acid into protein. This indicated that either vitamin B<sub>12</sub> is involved in the activation of the amino acid or the transference of it from the amino acid adenylate to S-RNA. It was found that hydroxamate formation by the normal and deficient pH 5-enzymes was the same<sup>23</sup>; this ruled out the possible role of the vitamin in the activation of the amino acids.

S-RNA isolated from the deficient incubation mixture was much less labelled than that isolated from the normal. This can be seen from the data summarised in Table IV.

This shows that vitamin B<sub>12</sub> is involved in the transferring step rather than in the activation step. That it is not a part of the activating enzymes is shown by Szafranski and others<sup>24</sup> very recently.

There is a possibility of the vitamin existing or rather functioning in a cofactor form in the animal body, since the cofactor could restore the incorporation of the amino acid in the deficient system almost to normal level<sup>23</sup>.



TABLE IV. Incorporation of 3-C<sup>14</sup>-phenylalanine into S-RNA and microsomal protein by pH 5-enzymes and microsomal system (respectively) of vitamin B<sub>12</sub>-deficient and normal rats

		Vitamin B <sub>12</sub> -deficient			Vitamin B <sub>12</sub> -normal		
		Total RNA in mg	cpm. in RNA	cpm./mg protein	Total RNA in mg	Total cpm. in RNA	cpm./mg Protein
pH 5-enzyme system	...	1.88	560	...	1.85	1820	...
	...	1.82	510	...	1.78	1780	...
Microsomal system	...	...	...	117	...	...	120

pH 5-enzymes system contained 2 ml of pH 5-enzymes (20 mg protein), 1.0  $\mu$ mole 3-C<sup>14</sup>-phenylalanine, 2.0  $\mu$ mole ATP, 40  $\mu$ moles PEP, 0.1 mg PK, and 5.0  $\mu$ moles MgCl<sub>2</sub>.

Microsomal system contained 0.4 ml of microsomes (10 mg protein), 0.5 mg of S-RNA-as (containing 1500 cpm.), 0.5  $\mu$ mole ATP, 0.25  $\mu$ mole GTP, 10  $\mu$ moles PEP, 0.05 mg PK.

A recent report by Arnstein and White<sup>25</sup> is very encouraging though the results seem to have been misinterpreted. It is quite evident that there is a lower incorporation of the labelled amino acids into protein of the deficient cultures of *Ochromonas malhamensis*.

It is time to tag up the exact function of vitamin B<sub>12</sub> and, perhaps, the exact site of its action. It seems quite clear that this vitamin is definitely involved in one of the steps of protein biosynthesis. Until and unless all the discrepancies in the results reported by different investigations are resolved, nothing could be said with certainty regarding the function of this vitamin.

### Acknowledgment

We are grateful to Dr W. H. Ott for the B<sub>12</sub> deficient and normal chicks and to Dr. C. Rosenblum of Merck and Sharp and Dohme for the generous gift of Co<sup>60</sup> labelled vitamin B<sub>12</sub>.

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### Discussion

- Q. How could the present results be reconciled with the contradictory findings reported by Arnstein and co-workers?
- A. Firstly, the divergences may arise through the employment of different amino acids whose rates of incorporation may vary. Secondly, and more importantly, the latter authors have used the free vitamin whereas we have used the cofactor form of the vitamin.



# ROLE OF VITAMIN B<sub>12</sub> IN PROTEIN SYNTHESIS BY *ESCHERICHIA COLI*

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The experiments reported in this communication were designed to find out whether vitamin B<sub>12</sub> plays any part in protein synthesis by *Escherichia coli*. The spectacular success achieved in unravelling the mechanism of vitamin growth inter relationship by employing mutant cultures of microorganisms suggested that a study of protein synthesis by a mutant strain of *Escherichia coli* with a specific requirement for the vitamin, might be worthwhile in gaining some insight into the mechanism of action of the vitamin in bacterial protein synthesis. Incorporation of labelled amino acids into the trichloroacetic acid precipitable proteins and induction of adaptive enzyme formation were used as measures of the extent of protein synthesis by the bacterium.

## Experimental Materials and Methods

The strain used in this study was a mutant of *Escherichia coli* M—200—12.13 originally isolated by Davis and Mingoli<sup>1</sup>. It requires vitamin B<sub>12</sub> for optimal growth in a minimal medium made up of mineral salts and glucose vitamin B<sub>12</sub> could be replaced by methionine, but several hundredfold concentration of the latter is required to give comparable growth to that given by as low as  $5 \times 10^{-4}$   $\mu$ g. of vitamin B<sub>12</sub>. The cells employed in the present experiment were obtained by inoculating a drop of a suspension of the culture (made by suspending a loopful of 18 hr growth on a nutrient agar slope in 10 ml sterile 0.85 per cent sodium chloride) into 25 ml of glucose-mineral salt medium supplemented with vitamin B<sub>12</sub> contained in a 250 ml Erlenmeyer flask. Effective aeration during incubation at 35° was provided by rocking the flasks in a cradle type shaker or in a rotary bottle shaker. Growth was followed at hourly intervals by measuring the turbidity of the suspension (Klett readings, Red filter 660 m $\mu$ ) and within two hours after growth had ceased, the cells were separated from the medium by centrifugation (1800 $\times$ g, 20 mts. at 5°C). The sedimented cells were washed twice by suspending in 20 ml portions of 0.05 M Sorenson's phosphate buffer pH 7.0 and centrifuging at 2000 g. for 20 mins. at 5°C. The cells were finally dispersed in buffered mineral salts medium.

## Results

*Synthesis of cellular constituents by Escherichia coli:* 5 ml of cell suspension, prepared as above, were transferred to flasks containing 5 ml of fresh mineral salts medium with and without vitamin B<sub>12</sub>. The flasks were shaken in a rotary bottle shaker and 1 ml aliquots withdrawn at intervals and added to 1 ml of cold 10 per cent trichloroacetic acid. The contents of the tubes were centrifuged and cell sediment extracted twice with 5 per cent trichloroacetic acid at 95° for 10 minutes. The trichloroacetic acid extracts were separated each time by centrifugation, combined and used for optical density measurements and



estimation of ribonucleic and deoxyribonucleic acids<sup>2,3</sup>. The protein pellets were dispersed in alkaline copper reagent and protein content estimated colorimetrically<sup>4</sup>. The results of a typical experiment are presented in Fig. 1. The RNA content falls sharply from 0 to 30 mins. and remains more or less at the plateau level for the rest of the incubation period. There is not much of a significant difference in the shapes of the curves with and without vitamin B<sub>12</sub>, excepting that there is an indication of a decline after 3 hours in the system without vitamin B<sub>12</sub>, whereas the level of the nucleotide remains constant and slightly rises at 4 hours in the system with vitamin B<sub>12</sub>. The corresponding curves of acid soluble 260 m $\mu$  absorbing materials do not exhibit this sharp fall from 0-30 mins. The two curves (with and without B<sub>12</sub>) run almost parallel upto 150 minutes after

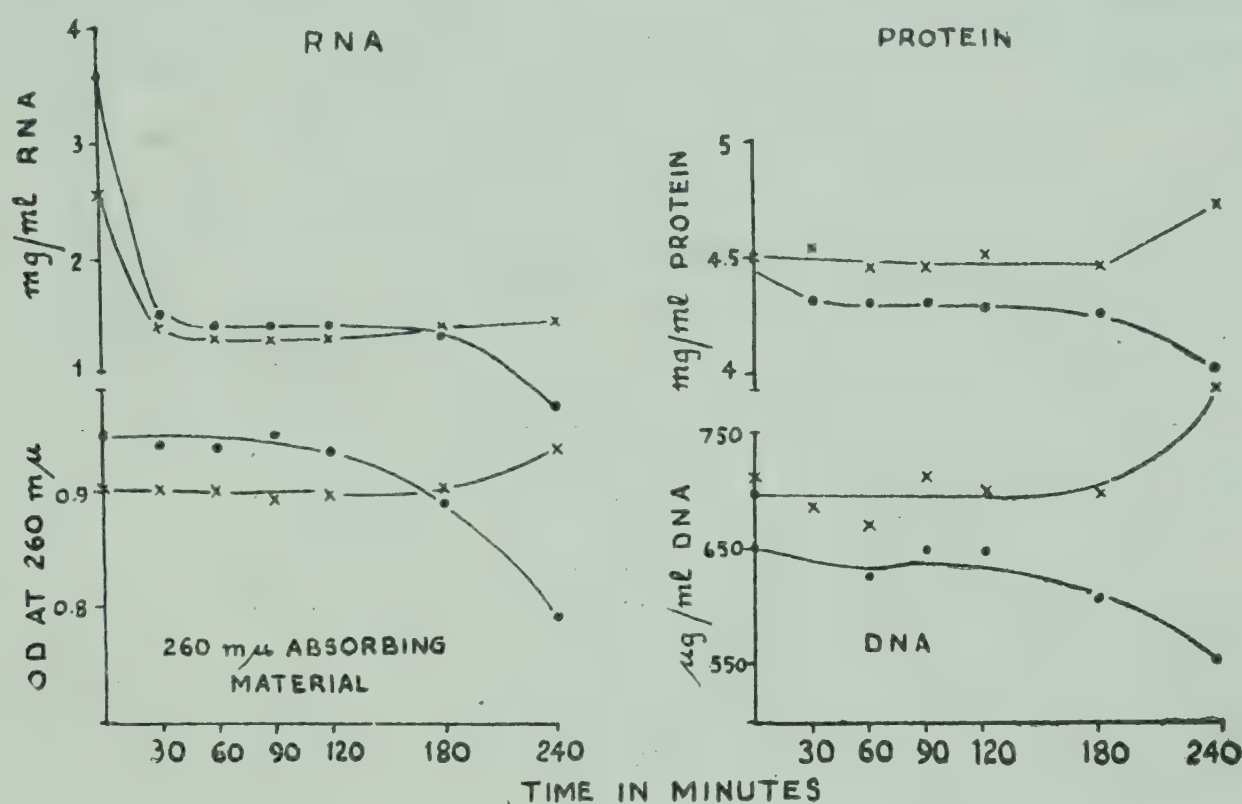


FIG. 1. Synthesis of cellular constituents by *E. Coli*

which the system without vitamin B<sub>12</sub> registers a fall. The curves for protein in the two systems exhibit, however, some difference. They run almost parallel upto 120 mins. The curve for the system without vitamin B<sub>12</sub> declines at 180 mins. corresponding to the fall in RNA content, whereas the protein curve for the system with vitamin B<sub>12</sub> remains unchanged upto 180 mins. but soon starts rising to a value higher than the plateau value of the system without B<sub>12</sub>. The curves representing DNA content in the two systems broadly resemble the protein curves. The results indicate that, under the conditions employed, multiplication occurs in the system with vitamin B<sub>12</sub> as clearly evident from the increase in DNA and protein contents, whereas there is no multiplication of cells in the system without vitamin B<sub>12</sub>. The results, however, do not permit any *a priori* conclusions to be drawn with regard to the question whether the growth factor influences protein synthesis or the stimulation of protein synthesis is an indirect result of stimulation of RNA and DNA synthesis. Answers to these questions were, therefore, sought by carrying out incorporation of radio-active amino acids into proteins and induction of adaptive enzymes. The two processes obviously involve protein synthesis and any effect of vitamin B<sub>12</sub> on these phenomena could be taken as evidence, one way or the other, for a direct participation of the vitamin in protein synthesis.



*Incorporation of leucine 1-C<sup>14</sup> into E. coli proteins:* 10 ml of cell suspension was mixed with 60 ml of 'incorporation medium' made up of 45.4 ml of 0.05 M phosphate buffer pH 7.0, 9.6 ml. carrier-diluted leucine 1-C<sup>14</sup> (approximately 10 counts per minute/ $\mu$ g) and 5 ml. of 20 per cent glucose. Concentration of bacterial cell was adjusted so as to give approximately 1 mg dry wt./ml of the final mixture. 20 ml of this reaction mixture were immediately pipetted into a 100 ml centrifuge tube containing 10 ml 20 per cent trichloroacetic acid and 2 ml water. 20 ml aliquots of the reaction mixtures were transferred to 250 ml Erlenmeyer flasks containing 2 ml water or 2 ml of vitamin B<sub>12</sub> ( $10^{-2}$   $\mu$ g). The two flasks were shaken vigorously to provide aeration at 35° for two hours and the proteins precipitated with 10 ml of 20 per cent trichloroacetic acid. The precipitated proteins were processed for plating and counting essentially according to the procedure described by Mandelstam<sup>5</sup>. The dry proteins were packed to infinite thickness in 0.3 sq. cm. cross area perspex discs and counted in an end window Dyanatron counter. The results are given in Table I.

TABLE I

*Radioactivity of proteins of Escherichia coli*

	Counts/minute corrected for background and thickness
Protein isolated at 0 time ...	4
Protein isolated after 2 hours of incubation in medium without vitamin B <sub>12</sub> ...	224
Protein isolated after 2 hours of incubation in medium with vitamin B <sub>12</sub> ...	210

The above experimental set-up would correspond to 'condition a 1' according to Gale.<sup>6</sup> Incorporation of radioactivity under these conditions presumably takes place as a result of an exchange reaction between the added labelled amino acid and corresponding residues in the protein already present in the preparation. The incorporation is brought about conjointly by proteins, amino acids and nucleic acids, and all inhibitors which interfere with incorporation also adversely affect protein synthesis. The exchange-incorporation is 'an activity displayed by a part or parts of the mechanism involved in protein synthesis'. The total count registered by the trichloroacetic acid precipitated proteins of cellular preparation can therefore be taken as a broad index of protein synthesis, even though only a single labelled amino acid is used in the medium. Under the conditions employed in this experiment, no significant differences could be noticed in the total activity of the isolated proteins, whether or not vitamin B<sub>12</sub> was included in the medium. In view of this, a direct implication of the vitamin in protein synthesis by *Escherichia coli* does not appear to be justifiable.

It is pertinent in this connection to refer to the work of Dinning *et al.*<sup>7</sup>, on *Lactobacillus leichmanii*. From incorporation studies with cellular preparations of the organism, no evidence could be adduced for the influence of vitamin B<sub>12</sub> in the utilization of labelled amino acids for purine or protein synthesis. In analogous experiments with the flagellar protozoan *Ochromonas malhamensis*, Arnetein and White<sup>8</sup> have, however, recently reported



a stimulatory effect of vitamin B<sub>12</sub> on the rate of incorporation of amino acids. The vitamin increased the specific radio-activity of the protein nearly two fold. Hydrolysis of the labelled protein, chromatography of the amino acids liberated and counting of activity in individual amino acids indicated that, in the absence of vitamin B<sub>12</sub>, labelling of certain amino acids like valine, methionine, tyrosine etc., was reduced much more than the rate of incorporation of the amino acids into protein. The conclusion drawn from this was that vitamin B<sub>12</sub> is intimately connected most with the biosynthesis of some amino acids rather than in actual protein synthesis confirming the observations of Ford and Goulden<sup>9</sup> in growth studies with the same organism. Presumably, the role of vitamin B<sub>12</sub> in *Ochromonas* in contrast with its role in *E. coli* and *L. leichmanii* is akin to its function in mammalian tissues.

*Induction of adaptive enzymes in E. coli:* The mutant of *E. coli* can adapt itself on lactose for growth and should, therefore, possess the inductive mechanism for the formation of  $\beta$ -galactosidase. This induction presumably involves two enzymes, *viz.*, a permease which allows the inducer lactose to gain access into the cell and, secondly, a  $\beta$ -galactosidase which hydrolyses the lactose to glucose and galactose. Since *E. coli* mutants do not synthesize inducible enzymes in the absence of the specific co-factor required for growth<sup>10</sup>, it was of interest to find out how far vitamin B<sub>12</sub> influences adaptation, on lactose, of the mutant under study. Induction experiments were carried out with resting cell suspensions made from acetate grown cells. In a typical experiment, the medium contained in a total volume of 10 ml washed suspension (10 mg dry wt.), 10  $\mu$  moles of lactose (inducer), 100  $\mu$  moles of sodium acetate and 0.1  $\mu$ g vitamin B<sub>12</sub> when added. The reaction mixtures were shaken vigorously at 37° and 1 ml aliquots withdrawn at intervals and pipetted into a solution containing 100  $\mu$  moles sodium dihydrogen phosphate (pH 7.2), 100  $\mu$ g chloramphenicol and 0.1 ml of toluene in a total volume of 1.6 ml. Presence of chloramphenicol effectively checked further synthesis of adaptive enzyme in the system. The tolunized cells were incubated with occasional shaking in a water bath at 37° to bring about release of endo-cellular  $\beta$ -galactosidase. The enzyme was assayed according to Kuby and Lardy<sup>11</sup> using 5  $\mu$  moles of O-nitrophenyl galactopyranoside per tube. The results presented in Fig. 2 indicate that the induction process is not affected by the presence or absence of the vitamin.

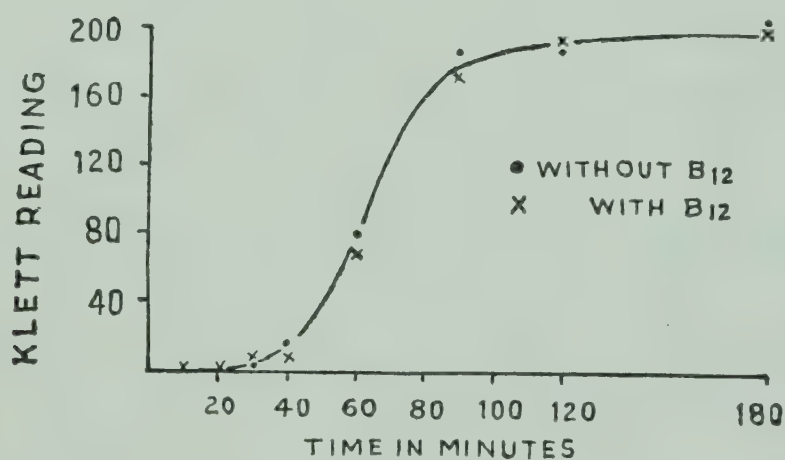


FIG. 2. Induction of  $\beta$ -galactosidase in *E. coli*.

In order to find out whether vitamin B<sub>12</sub> affects formation of permeases, rates of oxidation of acetate and succinate were studied with cells grown on glucose. Since chloramphenicol, a typical inhibitor of protein synthesis, inhibits adaptation, there is presumptive evidence to indicate that induction of permeases involves protein synthesis. Suspensions



of the mutant strain grown on glucose, however, readily oxidised acetate and succinate; similarly suspensions of cells grown on acetate readily oxidized succinate and malate ruling out the existence of permease systems for these substrates in the mutant strain of *E. coli*.

## Conclusions

The results presented in this study would thus exclude any possibilities of a direct participation of vitamin B<sub>12</sub> in protein synthesis by *E. coli*. The observations are in conformity with the view<sup>12</sup> that in B<sub>12</sub> requiring mutants the vitamin is apparently more concerned with the catalytic formation and transfer of methyl groups rather than in protein synthesis.

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## Discussion

Q. Is 'permease' a concept or an entity?

A. Recent work has shown that permeases are proteins with specific enzymic properties and serve to transport certain substrates into the cell.

Q. According to the original concept of Cohen and Monod, permeases are stereo-specific in the sense that while a particular metabolite depends for its entry into the cell on a specific permease, its stereo-isomer is taken up and utilized without the mediation of a permease. What was the specific permease studied in the above investigations?

A. While no specific permeases were estimated, inferences have been drawn depending upon the utilization of certain metabolites.

Q. Have any experiments been carried out using organisms adapted to methionine or grown on media containing methionine?

A. No.



# INTERDEPENDENCE OF RIBONUCLEIC ACID AND PROTEIN SYNTHESSES IN *SACCHAROMYCES CEREVISIAE*

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Since the suggestion that ribonucleic acid (RNA) is concerned with protein synthesis was presented independently by Caspersson<sup>1</sup> and Brachet<sup>2</sup>, many experiments which support that hypothesis have been reported. Gale and Folkes<sup>3</sup> reported that *Staphylococcus aureus* RNA, or its RNA digest, could support the incorporation of radioactive glutamate into protein when added to disrupted cells depleted of their RNA content. Brachet<sup>4</sup> and co-workers demonstrated similar effects by adding yeast RNA to ribonuclease (RNAase) treated amoebae. On the other hand, much experimental evidence<sup>5,6</sup> especially that concerning induced enzyme formation, suggests that protein synthesis requires not only the existence of RNA as a template but also concurrent RNA synthesis. However, not all the findings obtained using microorganisms support the idea of compulsory dependence of protein synthesis on co-synthesis of RNA<sup>7</sup>. During the past few years, the problem of concomitant renewal has been one of the foci in discussions concerning the role of RNA in the mechanism of protein synthesis.

Preliminary observations relating to the kinetics of protein and nucleic acid syntheses in *Saccharomyces cerevisiae* grown under conditions that would restrict the supply of precursors required for RNA build-up are presented in this paper.

## Experimental

Locally isolated strain of *Saccharomyces cerevisiae* was used in these experiments. Cells were grown aerobically for 18 hr in a medium consisting of bactopectone 10, yeast extract 1.0 and glucose 40 g./l. These were washed several times with water before being used for further study. All the experiments were carried out under aerobic conditions.

**Phosphate starvation:** This was carried out in a medium (-P) composed of glucose 40, a mixture of 18 amino acids simulating the composition of yeast protein equivalent<sup>8</sup> to nitrogen 0.8, MgSO<sub>4</sub> 0.09, Na<sub>2</sub> SO<sub>4</sub> 0.13 g./l. The initial density of the culture was 0.6 g./l. Samples were removed frequently during a period of 20 hr and analysed for cell count, protein and RNA.

**Phosphate replenishment:** Cells subjected to 20 hr phosphate starvation were withdrawn and then grown in the same volume of fresh synthetic medium (-P) supplemented with KH<sub>2</sub> PO<sub>4</sub> 3 g./l. Samples were removed frequently during a period of 4 hr and analysed for cell count, protein and RNA.

**Aminopterin inhibition:** Eighteen hr cells grown in peptone medium were suspended in the synthetic medium (-P) containing KH<sub>2</sub> PO<sub>4</sub> 3 mg/ml and aminopterin 1.7 µg/ml. Protein and nucleic acid syntheses were followed up for a period of 12 hr.

**Glycine adaptation:** The adaptation was effected by successive transfers of yeast cells on the synthetic medium with the composition (g/l): glucose 40, glycine 3, NaCl 3, MgSO<sub>4</sub> 0.09, Na<sub>2</sub> SO<sub>4</sub> 0.13 and KH<sub>2</sub> PO<sub>4</sub> 0.15. Adapted cells were first grown on this

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medium for 18 hr. Phosphate starvation was effected in the same medium without phosphate, the initial density of culture being 0.6 g./l. Protein and nucleic acids were determined in the samples removed at frequent intervals during 16 hr.

*Labelled experiments:* Labelled experiments were performed with (i) peptone grown yeast cells prior to phosphate starvation and (ii) those phosphate starved for 12 hr; at this stage, a little breakdown of RNA occurred. Each batch of cells was equally distributed in two media to a density of 4 mg dry wt. per ml. One medium composed of  $5.4 \times 10^4$  cpm glycine -2-C<sup>14</sup> per ml of the synthetic medium (-P). The other contained in addition to this 0.3 per cent KH<sub>2</sub>PO<sub>4</sub>. Incubation was carried out for 10 minutes and the cells were then immediately fractionated for protein and nucleic acids, according to the method of Schneider<sup>9</sup>, into cold trichloroacetic acid (TCA) soluble, hot TCA-soluble and hot TCA-insoluble fractions. The protein fractions were dissolved in alkali, reprecipitated, washed with 5 per cent TCA and redissolved in 10 per cent NaOH. The activities in the fractions were determined directly at infinite thinness on glass planchets employing the Tracerlab SC-16 windowless gas-flow counter in conjunction with the Tracerlab SC-51 autoscaler.

*Protein and RNA estimation:* The cells were fractionated according to Schneider's method<sup>9</sup>. RNA in the hot TCA-fraction was estimated by the colour reaction with orcinol.<sup>10</sup> Protein in the hot TCA-precipitate was estimated by micro-digestion and Nesslerisation.<sup>11</sup> Cell count was done by means of a haemocytometer.

*RNAase estimation*<sup>12</sup>. RNAase was estimated by E<sub>260</sub> measurements of hydrolysed RNA. The reaction mixture composed of 0.5 per cent RNA (Schwarz) in 0.1 M acetate buffer pH 5.0; 0.1 per cent yeast preparation. Incubation was carried out for 1 hr at 30° C. A change in O.D. of 0.01 was taken as one enzyme unit under these conditions.

## Results

During phosphate starvation of yeast cells grown previously on peptone medium, both protein and nucleic acids were synthesised for a period of 8 hr. The synthesis of RNA was affected drastically thereafter and the breakdown ensued. Protein content, however, continued to rise in spite of the absence of concomitant increase in RNA. The rate of increase in cell number paralleled the protein renewal (Figure 1).

The cells subjected to severe phosphate starvation (20 hr) showed a marked difference between the rates of synthesis of these two macromolecules when grown in phosphate containing medium. An initial lag of 2 hr in protein synthesis was observed, although RNA synthesis was resumed as soon as phosphate was supplied (Figure 2). This synthesis of RNA was completely inhibited by the amino acid analogue p-fluorophenylalanine, at a concentration of 0.1 M. in the medium.

Some inhibitors known to interfere selectively with either protein or nucleic acid synthesis were tried in order to obtain disengagement of the two processes. Among these, aminopterin is effective in completely inhibiting RNA synthesis, whereas protein renewal continues thereafter although at a lower rate. Resumption of RNA synthesis occurs after 6 hr, possibly through enzymic inactivation of aminopterin.<sup>13</sup> Protein synthesis, however, is not immediately accelerated simultaneously with initiation of RNA synthesis but shows some lag before being restored to normal (Figure 3). The results with aminopterin resemble the pattern of relative syntheses of protein and nucleic acids as obtained in phosphate starvation and subsequent phosphate replenishment.



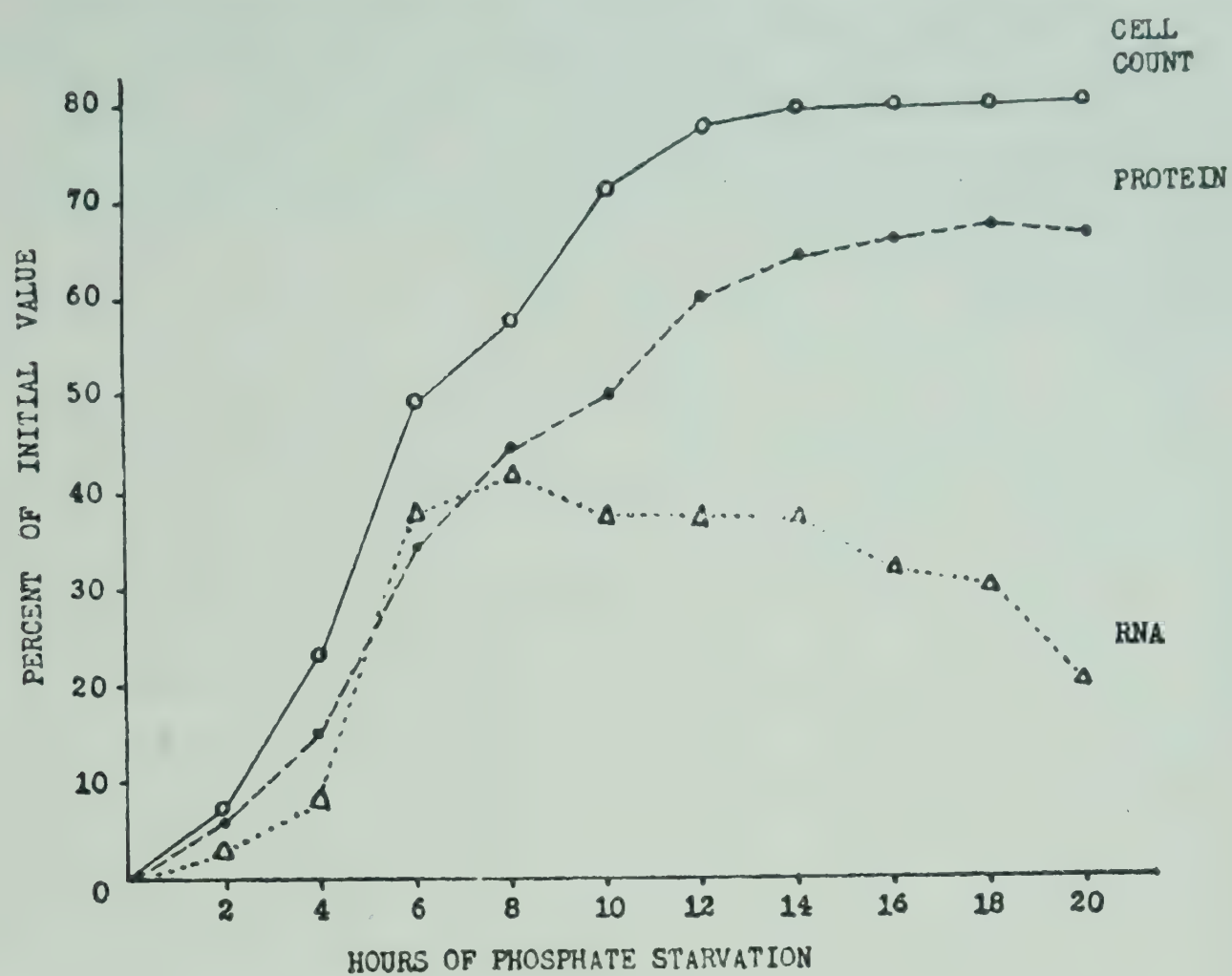


FIG. 1. Phosphate Starvation of *S. Cerevisiae*: Cell Count, Protein and RNA Increments

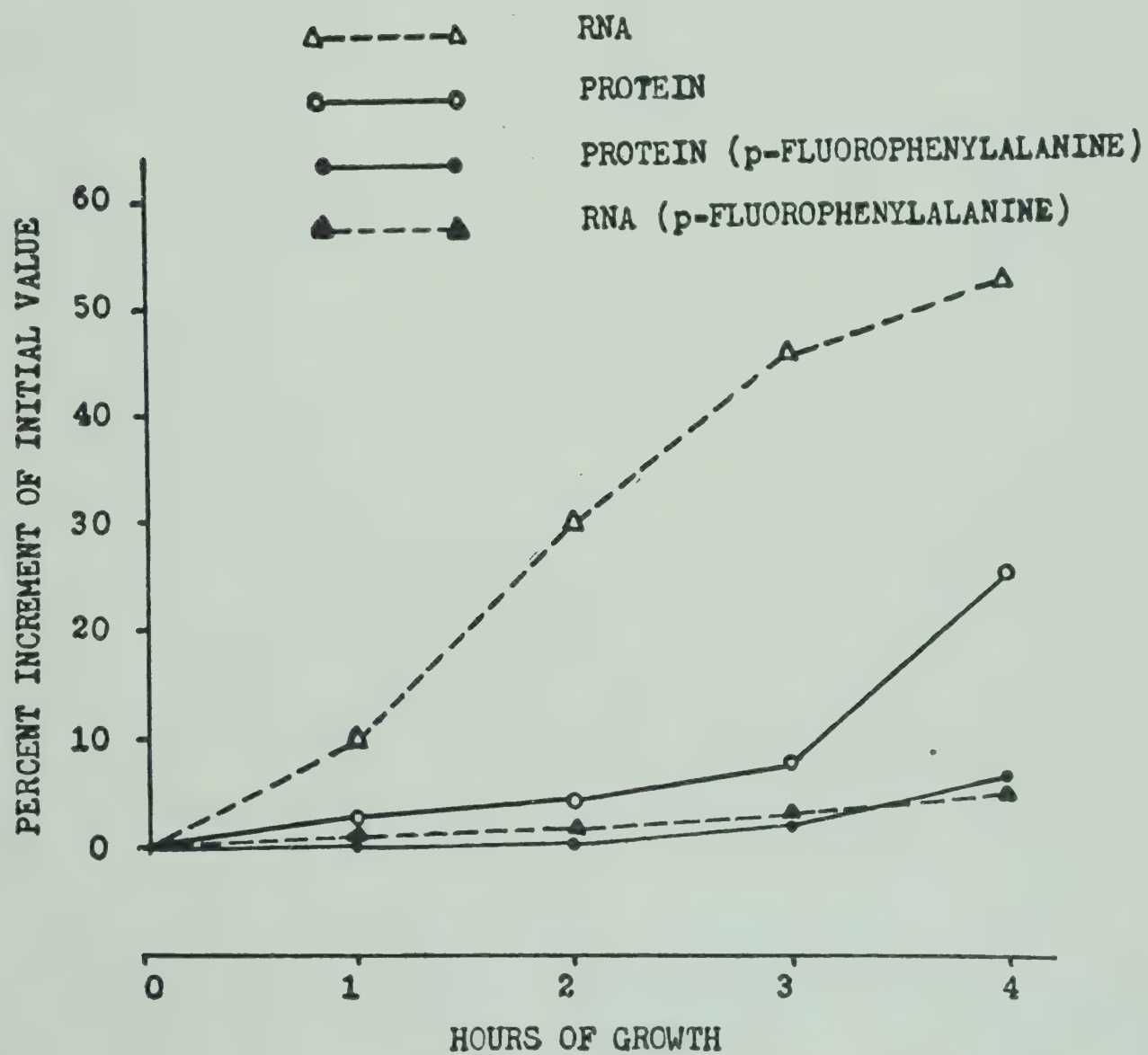


FIG. 2. Phosphate Replenishment of Phosphate Starved *S. Cerevisiae*: Protein and RNA Increments



Although an uncoupling of the two syntheses is thus indicated in phosphate starvation and aminopterin inhibition, a possibility of RNA turnover at this stage was not precluded. After phosphate starvation on amino acid medium for 12 hr (uncoupling stage), the yeast cells were examined for their ability to incorporate glycine-2- $C^{14}$  into protein and nucleic

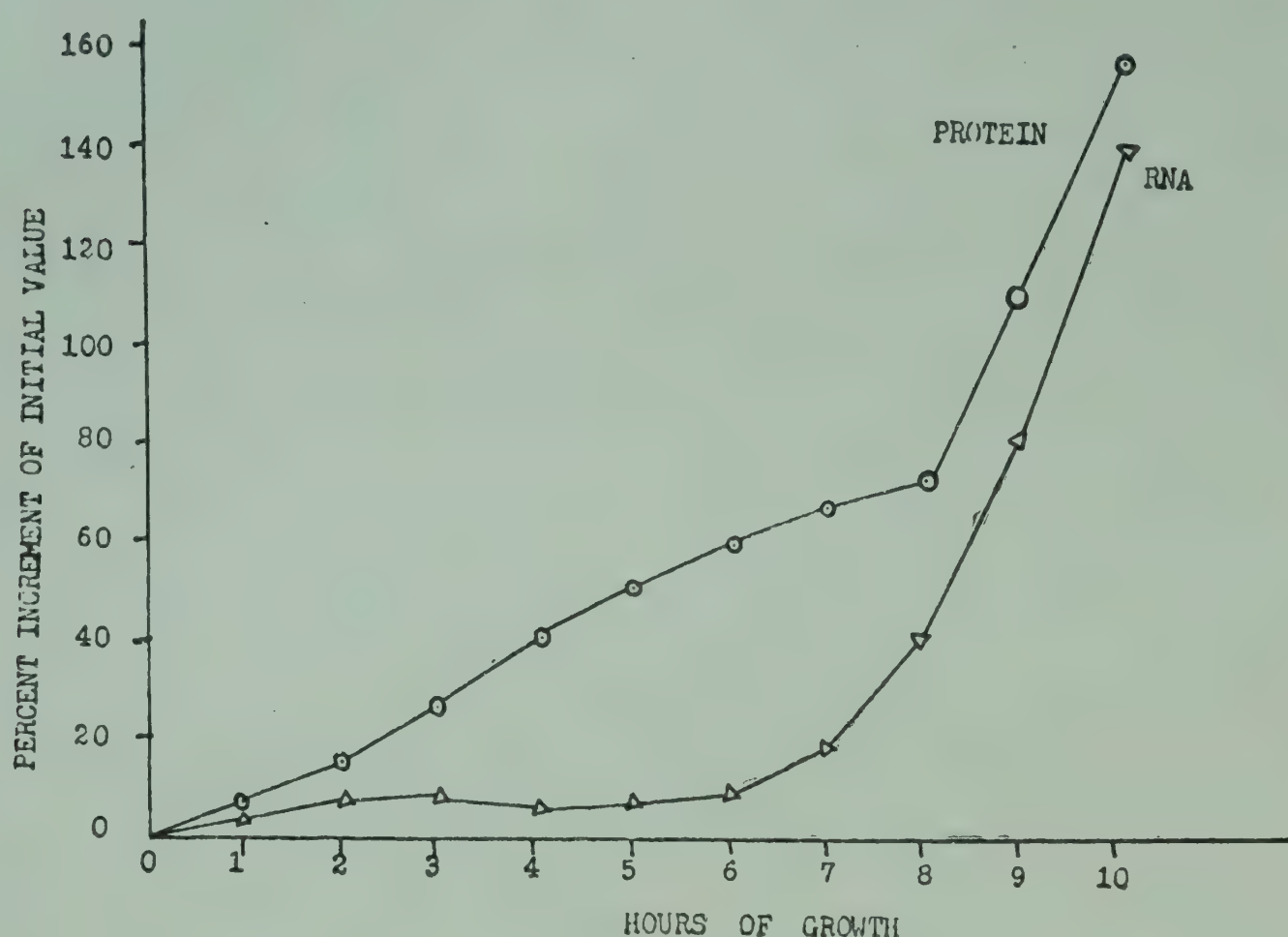


FIG. 3. Aminopterin inhibition of *S. Cerevisiae*: RNA and Protein Increments

acids during exposure for 10 min. From Table I, it is clearly seen that RNA renewal does occur at the stage where there is no net rise in RNA accompanying net increment in protein content. According to Halvorson<sup>14</sup>, such an incorporation, at least with labelled glycine, into yeast protein and nucleic acids, reflects true synthesis rather than a consequence of exchange reactions. The incorporation into protein and nucleic acids by phosphate starved yeast cells is lower than that by normal cells. Inclusion of phosphate in

TABLE I

*Incorporation of glycine- 2-  $C^{14}$  into protein and nucleic acids of *S. Cerevisiae* (10 minute incubation)*

	Normal cells		Phosphate starved cells	
	-P	+P	-P	+P
Counts per minute				
Nucleic acids ...	4250	4280	3205	4200
Protein ...	4995	5001	3970	4900

P = Phosphate as  $KH_2PO_4$  in the incubation system.



the incorporation system stimulates labelling of protein and RNA of the phosphate starved cells almost to the normal level. No stimulatory effect is demonstrable in peptone grown yeast cells.

The decrease in RNA during phosphate starvation prompted a study of RNAase activity of the culture. From Table II, it will be seen that the RNAase activity per cell progressively increases during phosphate starvation and is almost double the original value at the end of 16 hr.

TABLE II  
*RNAase activity of S. Cerevisiae: Effect of Phosphate Starvation*

Hours of phosphate starvation	Cell count per ml culture	Protein $\mu\text{g}$ per cell	RNA $\mu\text{g}$ per cell	RNAase units per cell
	$\times 10^{-8}$	$\times 10^{-8}$	$\times 10^{-8}$	$\times 10^{-8}$
0	1.50	390	78	20
4	1.68	353	78	23
8	2.12	360	78	26
12	2.42	430	73	34
16	2.65	405	59	41

Dissociation of the syntheses of protein and RNA is totally absent in the yeast cells adapted to utilise glycine as the sole source of nitrogen (Figure 4). When these cells are subjected to phosphate starvation in the glycine medium devoid of phosphate, syntheses of protein and nucleic acids continue for 8 hr. This is followed by simultaneous breakdown of the two moieties.

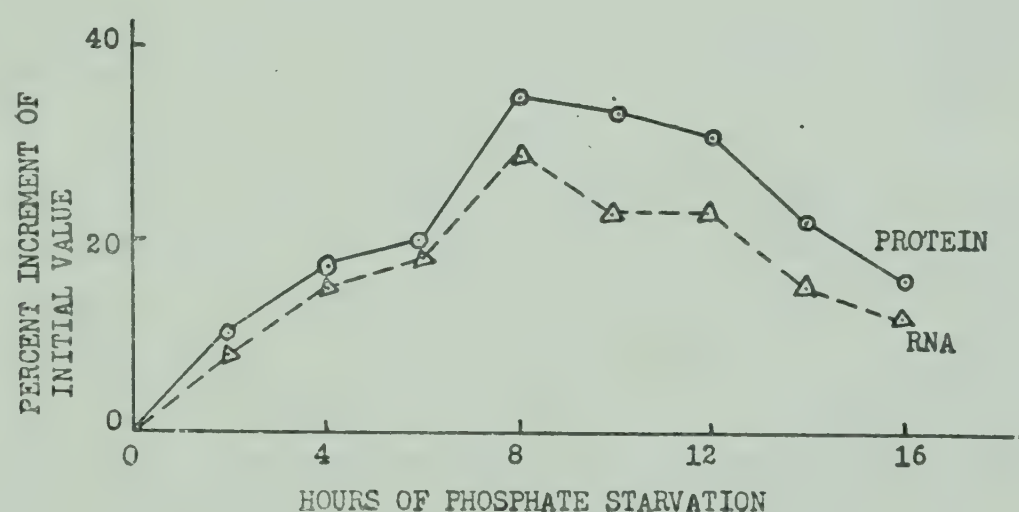


FIG. 4. Phosphate Starvation of Glycine-adapted Cells of *S. Cerevisiae*: Protein and RNA Increments

## Discussion

Kinetic studies of protein and RNA syntheses both in phosphate deprivation and in aminopterin inhibition indicate that large amounts of protein could be formed without simultaneous increment in RNA. At first sight, therefore, these findings suggest no direct requirement of co-synthesis of RNA for the protein synthesis. Barner and Cohen<sup>15</sup> demonstrated that, though net protein and adaptive enzyme formation could occur when total nucleic acid synthesis is inhibited in an *Escherichia coli* double mutant requiring uracil and thymine, a turnover of nucleic acids takes place under these conditions.

Labelled studies carried out with glycine -2-C<sup>14</sup> at the uncoupling stage during phosphate starvation clearly shows that new RNA is being formed at the expense of RNA



degraded during phosphate starvation to meet the requirement for new protein formation. The protein synthesis during aminopterin inhibition may presumably be accompanied by RNA turnover.

It has been shown in a number of independent studies that in actively growing cultures of yeast<sup>16</sup> and bacteria<sup>17</sup>, there is little, if any, breakdown of protein and RNA. By contrast, it has been found that in microbial cultures subjected to nitrogen or carbon starvation, there is a balance between degradation and resynthesis of these macromolecules. Such stress conditions necessitate elaboration of nucleases and proteolytic enzymes in order to regulate the economy of precursors of protein and nucleic acids. Appearance of RNAase activity under conditions of phosphate starvation could then be expected. In our studies with *Escherichia coli*, it was observed that elaborations of phosphatase and RNAase activities are instances of *de novo* synthesis of proteins (unpublished).

Events occurring soon after replenishing phosphate starved cells and reversal of aminopterin inhibition resemble the pattern of macromolecular build-up in cell population growing in the lag-phase. Accumulation of RNA and lag in protein synthesis in this growth phase is well known<sup>18</sup>. All these observations emphasize a need for preformed RNA as well as its co-synthesis for the new protein formation. Although certain independence of RNA formation from the concomitant protein synthesis could thus be inferred, its inhibition by p-fluorophenylalanine supports the general view that RNA formation is possible only under conditions favourable for protein synthesis.

These observations lend support to the hypothesis of a common precursor for both protein and nucleic acids<sup>18</sup>. Nevertheless, uncoupling stages under a wide variety of nutritional conditions must be sought and examined for macromolecular turnovers before any final conclusions could be arrived at. Also examination of cellular sites with respect to conjointly synthesised protein and RNA would reveal whether any direct interlinking of the two syntheses really exists or whether such a situation is a manifestation of balanced growth.

The dissimilarity in the behaviour of peptone grown and glycine adapted cells with respect to protein degradation may be attributable to varying liability of their macromolecules. Such a possibility could be considered in the light of the suggestion<sup>19</sup> that proteins and probably nucleic acids differ in their stability towards degradation. Thus it has been reported<sup>20</sup> that induced enzymes are lost in the earlier stages of N-starvation of *Escherichia coli*. Relative instability of protein formed during glycine adapted growth could be considered in this context. Alternatively, early degradation of protein simultaneously with RNA breakdown might simply be the consequence of a change in order of appearance of proteolytic enzymes and RNAase; both these activities might have been increased simultaneously in the course of phosphate starvation of glycine adapted cells causing protein and RNA degradation at the same time.

## Summary

(i) Kinetics of protein and nucleic acid syntheses of peptone grown yeast cells have been examined in phosphate starvation and aminopterin inhibition.

(ii) In both these cases, a stage was found to be reached where protein synthesis continued without any net increment in ribonucleic acid. There is an increase in RNAase activity per cell during phosphate starvation.



(iii) With the use of glycine -2-C<sup>14</sup>, it was revealed that uncoupling of the syntheses as observed in phosphate starvation is only apparent and that a turnover of RNA accompanies protein synthesis.

(iv) Resumption of RNA synthesis immediately after phosphate replenishment of phosphate starved cells or after reversal of aminopterin inhibition is associated with a lag in protein synthesis. RNA synthesis after phosphate replenishment is, however, inhibited by the amino acid analogue, p-fluorophenylalanine.

(v) Phosphate starvation of glycine adapted cells causes degradation of protein and RNA at the same time and no dissociation of the two syntheses is observed.

## Acknowledgment

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## Discussion

- Q. What was the effect of phosphate starvation on the viability of the cells? Were viable cell counts done?
- A. While viable cell counts were not done, it may be stated that the phosphate starvation was not prolonged to the stage of killing off or affecting the viability of the cells.



# THE RELATIONSHIP OF INDUCED CATALASE SYNTHESIS TO RIBONUCLEIC ACID METABOLISM IN YEAST\*

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Since the demonstration by Gale and Folkes<sup>1</sup> of the dependence of enzyme synthesis in disrupted cells on nucleic acids, it has been recognized that the relationship is either of two kinds. In one, the enzyme formation is dependent on the preformed ribonucleic acid; in the other, exhibited by adaptively formed enzymes, the synthesis is dependent on the simultaneous build up of RNA<sup>2,3</sup>.

The present work relates to the induced appearance of active catalase on aeration of yeast cells grown under anaerobic conditions<sup>4</sup>, particularly in its relation to nucleic acid metabolism.

## Experimental Materials and Methods

The organism used was a locally isolated strain of *Saccharomyces cerevisiae*, maintained by bi-weekly transfer on agar slants containing glucose 2 per cent, Bacto-peptone 1 per cent, yeast extract 0.3 per cent and agar 2 per cent.

For experimental purposes, the organism was grown in a broth of the same composition (without agar) for 20 hours at 30°C. Practically anaerobic conditions were maintained by using 500 ml Erlenmeyer flasks filled almost to capacity. Cells were harvested by centrifugation, washed with ice cold water and used at once.

The organism also grew well on a synthetic medium consisting of  $\text{NH}_4\text{Cl}$  1 per cent, glucose 1 per cent,  $\text{KH}_2\text{PO}_4$  1 per cent,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.01 per cent, and  $\text{CaCl}_2$  0.01 per cent. This medium was used after omission of  $\text{NH}_4\text{Cl}$  as a non-growth medium in the aeration experiments. The pH of the medium was adjusted to 6.8.

About 150 mg dry weight yeast cells were suspended per 10 ml of this medium and aerated in 50 ml Erlenmeyer flasks by shaking in a reciprocating shaker at the rate of 60 oscillations per minute. The cells were then harvested, washed with distilled water and resuspended in water. The cells were ruptured by repeated freezing and thawing and the catalase activity of the disrupted cells was assayed according to a procedure based on the titanium-colour reaction for hydrogen peroxide<sup>5</sup>, and developed in this laboratory by Manjrekar and Sreenivasan (unpublished).

To 10 ml of buffered (phosphate pH 6.8) hydrogen peroxide (M/800) were added 2 ml of the ruptured cell suspension and the whole was maintained at 0°C. 2 ml portions were removed at intervals and added to test tubes containing 5 ml lots of the titanium reagent. This served to stop the enzymic reaction and the stable yellow colour produced was proportional to the residual hydrogen peroxide, and was measured in a Klett-Summer-son photoelectric colorimeter with 420 m $\mu$  filter. Catalase activity was expressed in terms

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of 'Kat f'<sup>6</sup>, where  $\text{Kat f} = \frac{L X_o / X_t}{e} \times 10^2$  in which  $X_o$  and  $X_t$  are the residual hydrogen peroxide concentrations at 0 and t minutes and 'e' is the concentration of the enzyme source in the reaction mixture.

The yeast cells were fractionated according to the procedure of Schneider<sup>7</sup> into (i) cold 5 per cent TCA extract, (ii) hot 10 per cent TCA extract of the residue of (i) containing nucleic acids, and (iii) residue from (ii) dissolved in 10 per cent NaOH. The acid soluble nucleotides and the nucleic acids were estimated respectively in the cold and hot TCA extracts using their characteristic absorption maximum at 260 m $\mu$  ( $E_{260}$ ). Protein in the residue was determined according to the method of Lowry<sup>8</sup> *et al.* Cell multiplication during aeration was ascertained in terms of increase in turbidity measured in a Klett-Summerson photoelectric colorimeter with 660 m $\mu$  filter after diluting the culture 20 times.

## Results

*Aeration and appearance of catalase activity:* Using the N-free non-growth medium, the effect of the extent of aeration on the appearance of the enzyme activity was determined (Table I). The activity increased up to a period of 4 hours after which there was a gradual decline. In subsequent studies, this period was employed for induction of the enzyme. There was only a slight increase in cell mass and protein during the aeration. The RNA, on the other hand, showed a slight decrease during the period.

TABLE I  
*Effect of degree of aeration on catalase induction*

Time of aeration (hr)	Catalase activity (Units/ml)	RNA (mg/ml)	Protein (mg/ml)	Growth index (Klett Units)
0	28	0.94	3.36	98
$\frac{1}{2}$	123	0.94	3.20	98
1	155	1.04	3.52	102
2	175	1.04	3.84	106
3	210	0.98	3.84	106
4	240	0.92	3.86	106
5	235	0.92	3.90	106
6	230	0.92	3.92	106
7	230	0.91	3.92	106

Cells grown anaerobically in glucose-peptone-yeast extract medium for 20 hours, were harvested, washed and aerated for indicated periods in the non-growth nitrogen-free medium. Other details as in text.

Omissions of single constituents from the medium were made in order to fix the qualitative requirements for enzyme appearance. It was observed (Table II) that the presence of glucose was essential and the deletion of phosphate reduced the enzyme activity markedly. It was further seen that glucose could be substituted by other utilizable carbon compounds, particularly citrate and succinate.

*Influence of phosphate starvation:* The decline in enzyme activity when  $\text{KH}_2\text{PO}_4$  was omitted from the medium would point to the dependence of enzyme appearance on



TABLE II  
*Effect of medium constituents on catalase induction*

Omission from the medium	Catalase activity (Units/ml)
None	358
Glucose	144
KH <sub>2</sub> PO <sub>4</sub>	84
MgSO <sub>4</sub>	325
CaCl <sub>2</sub>	325

Cells obtained as described in Table I were aerated for 4 hours in the non-growth medium with omissions as indicated. Initial catalase activity was 27 units. Other details as in text.

phosphate. The small activity noticed in the absence of phosphate could be due to some phosphate carry-over, which possibility was next studied.

Cells harvested from a 20 hour culture in the glucose-bacto-peptone-yeast extract medium were suspended in a phosphate-free synthetic medium consisting of glucose 2 per cent, NH<sub>4</sub>Cl 0.2 per cent, NaCl 0.3 per cent MgSO<sub>4</sub> 0.009 per cent and Na<sub>2</sub>SO<sub>4</sub> 0.013 per cent and incubated at 30°C. under practically anaerobic conditions as described earlier. Occasional samples were tested after aeration for 4 hours for catalase activity. It was observed that if phosphate starvation was continued beyond 24 hours, there was rapid loss in appearance of catalase activity in aerated cells when phosphate was not supplied (Fig. 1). With phosphate starved cells there is a net decrease in the RNA content during aeration, and more so in cultures aerated in phosphate supplemented medium than in the phosphate-free medium.

*Relationship to nucleic acids:* The changes in catalase activity on aeration of phosphate-starved and fed cells do not bear any obvious relation to the changes in RNA. In order to verify whether a turnover of the nucleic acids was occurring simultaneously, two series of experiments were carried out. In one, the effect of certain analogues of uracil and guanine on enzyme formation during aeration of cell suspensions was studied. In the other, the incorporation of glycine 2-C<sup>14</sup> into RNA during aeration of the cell suspension was studied (Table III).

The results revealed a complete inhibition of catalase synthesis by 8-azaguanine at 200 µg/ml. Bromo-uracil and nitro-uracil at 500 µg/ml each depressed the enzyme formation by about 30 per cent. There is a marked labelling of the RNA as well as protein fractions when glycine 2-C<sup>14</sup> was added at start of aeration.

The possibility of overcoming the inhibition of catalase formation by 8-azaguanine was tried using purines and a partial hydrolysate of yeast RNA. The RNA was hydrolyzed by autoclaving with NH<sub>4</sub>OH at pH 11 at 10 lb. steam pressure for 4 hours. The hydrolysate was cooled, neutralized to pH 6.8 using 1 N HCl, made to volume and filtered.

Adenine, guanine or adenylic acid at a concentration of 200 µg/ml are ineffective in overcoming the 8-azaguanine inhibition of catalase synthesis. However, the RNA-hydrolysate, known to contain nucleosides, brings about significant reversal of the inhibition. This reversal was ascertained by adding the reversing agent at different time intervals



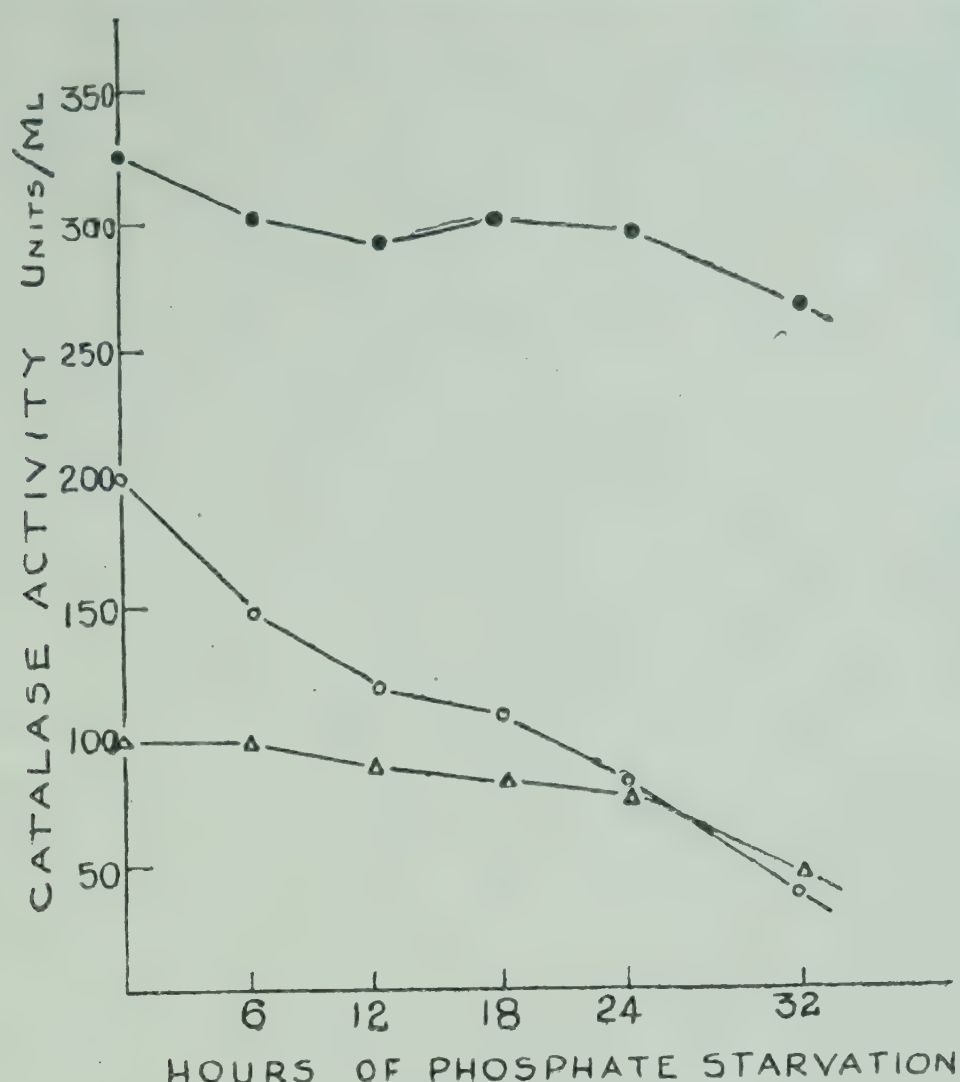


FIG. 1. Effect of  $\text{PO}_4^{+++}$  starvation on the ability to induce catalase in yeast

Cells starved of phosphate as described in text were aerated for 4 hours with and without phosphate.

Δ-Δ Initial activity; ○-○ Activity without phosphate; ●-● Activity with phosphate.

TABLE III  
*Incorporation of Glycine-2- $\text{C}^{14}$  into protein and RNA during catalase induction*

Aeration (hr)	Catalase activity (Units/ml)	Growth Index (Klett Units)	Protein		RNA	
			c.p.m./ml.	mg/ml.	c.p.m./ml.	mg/ml.
0	24	69	0	1.15	0	0.420
$\frac{1}{2}$	57	71	4000	1.2	2640	0.420
1	110	71	4880	1.2	3120	0.400
2	197	71	5320	1.2	3400	0.378
4	313	72	5900	1.2	4000	0.370

Cells obtained as described in Table I were aerated for 4 hours in the nitrogen-free medium with  $1.1 \mu$  moles of glycine ( $3.34 \times 10^4$  c.p.m.) per ml cell suspension. Other details as in text.

(Fig. 2). The delay between the addition of the reversing agent and of the inhibitor determined the degree of reversal.

In order to verify the effect of the preformed specific RNA that might be concerned in this synthesis, the anaerobically grown cells were exposed under anaerobic condition to an RNA rich extract<sup>9</sup> of cells grown aerobically. This treatment, however, proved ineffective. In fact, in aerated cultures this extract actually inhibited the appearance of the enzyme.



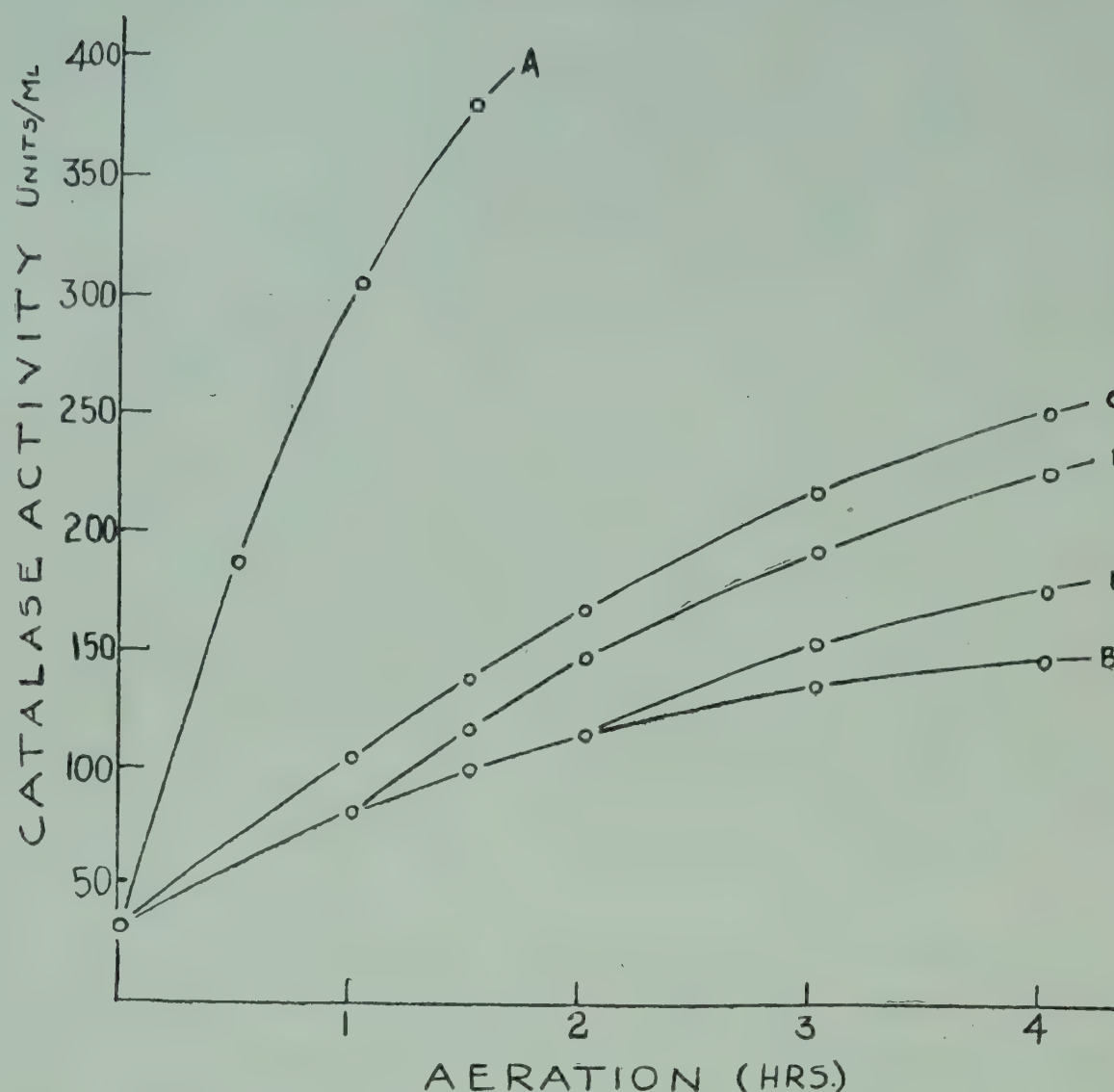


FIG. 2. Reversal of 8-azaguanine inhibition by RNA hydrolysate. Cells obtained as described in Table I were aerated for 4 hours.

A—No azaguanine added

B—Azaguanine added at 0 hours

C, D and E—RNA hydrolysate added at 0, 1, 2, hours after the start of aeration with 8-azaguanine.

*Effect of nitrogenous materials:* The RNA extract added was not free from non-RNA nitrogenous constituents. The inhibition may therefore be due to either of them. When casein hydrolysate<sup>10</sup>, or an amino-acid mixture, approximately simulating the composition of the yeast protein<sup>11</sup>, was added to the aerating medium, enzyme formation was again suppressed. These results are presented in Table IV. The inhibition by casein hydrolysate

TABLE IV  
Effect of RNA, amino acid mixture and casein hydrolysate on catalase induction

Addition to aeration medium		Growth Index (Klett Units)	Catalase activity (Units/ml) Increase in 4hr	Inhibition (Per cent)
None	...	50	288	...
RNA	...	67	170	41
Amino Acid mixture	...	75	205	28.7
Casein hydrolysate	...	82	190	34

Cells were grown anaerobically in glucose-peptone-yeast extract medium for 20 hrs. They were then harvested, washed and aerated for 4 hours in the non-growth nitrogen-free medium. All additions were at 5 mg/ml. Initial catalase activity was 27 units. Other details as in text.



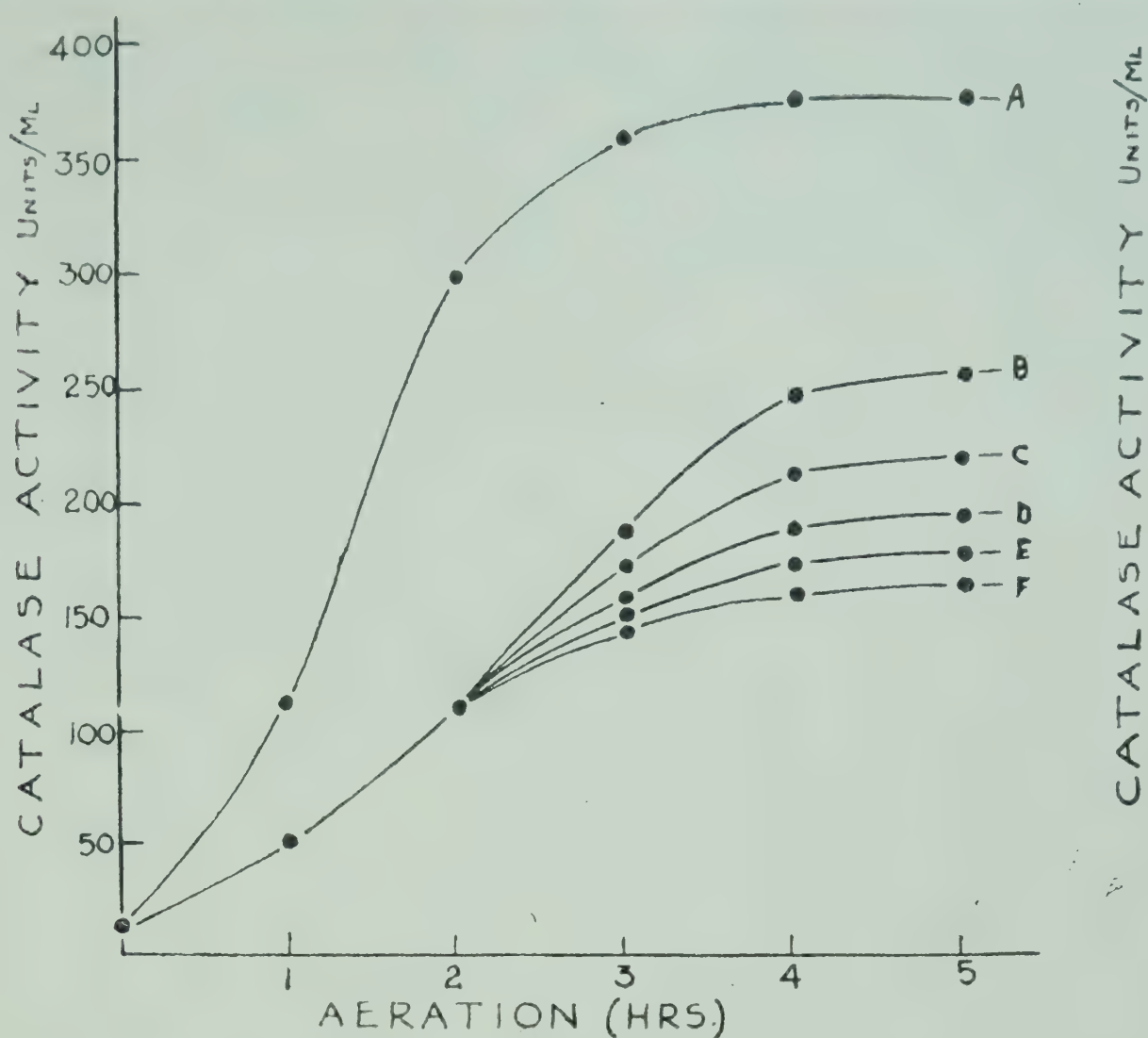


FIG. 3. Effect of different concentrations of casein hydrolysate on catalase induction.

Cells obtained as described in Table I were aerated for 4 hours.

A—No casein hydrolysate added.

B, C, D, E and F—Casein hydrolysate added at 0.5, 1, 1.5, 2 and 2.5 per cent level.

increased with concentration (Fig. 3) and was noticeable even when added at later stages of the aeration period (Fig. 4).

*Effect of cysteine:* Among individual amino acids, cysteine, but not cystine, at 2 mg/ml was found to be very inhibitory. When added together with casein hydrolysate, cysteine was less inhibitory than when present alone in the aeration medium.

Cysteine might act as an inhibitor by chelating an essential metal ion. The effect of a known chelating agent EDTA, not containing a sulphhydryl group, was therefore tried. EDTA inhibits catalase formation though at relatively high concentrations (Table V).

TABLE V  
Effect of casein hydrolysate, cysteine and EDTA on catalase induction

Addition to aeration medium	Growth Index* (Klett Units.)	Catalase activity (Units/ml.) Increase in 4 hr.	Inhibition (Per cent)
None ...	76	260	...
Cysteine ...	82	60	77
Casein hydrolysate ...	103	132	49.5
Cysteine + Casein hydrolysate ...	98	106	59.5
Cystine ...	82	260	...
EDTA ...	76	82	68.5

The additions were: Cysteine and cystine at 2 mg/ml, Casein hydrolysate at 5 mg/ml and EDTA at 20 mg/ml. Other details were as in Table I. Initial catalase activity was 25 units.



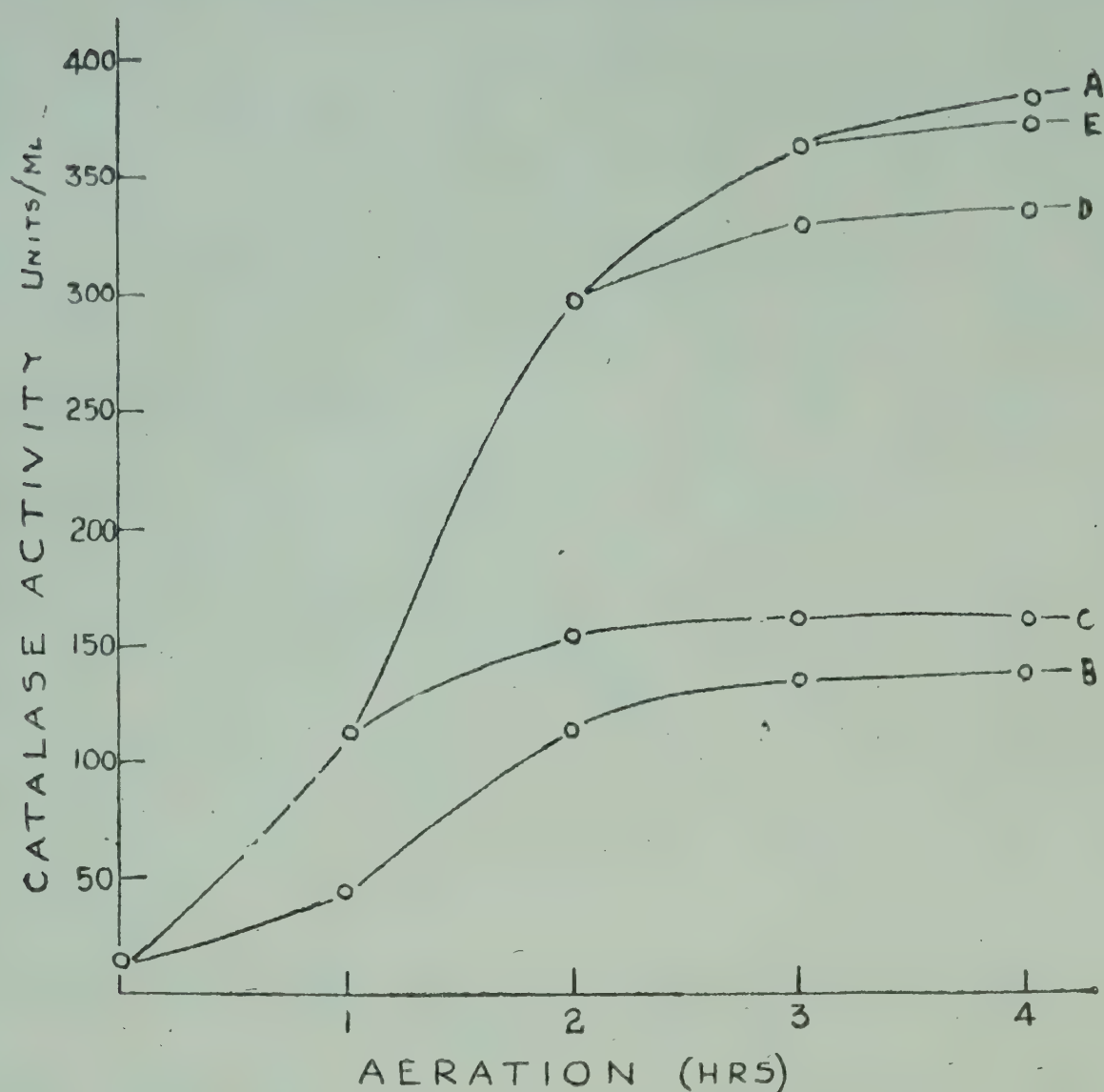


FIG. 4. Effect of casein hydrolysate on catalase induction. Cells obtained as described in Table I were aerated for 4 hours with 0.5 per cent casein hydrolysate.

A—No casein hydrolysate added

B, C, D and E—0.5 per cent casein hydrolysate added at 0, 1, 2 and 3 hours after the start of aeration.

*Reversal of inhibition by  $Fe^{+++}$* : Iron was added as Ferric ammonium citrate at a concentration of 1 mg/ml and reversed the inhibition due to cysteine or EDTA, but was not as effective with casein hydrolysate inhibition. Iron by itself did not interfere with catalase formation (Table VI).

TABLE VI

*Reversal by  $Fe^{+++}$  of inhibitions of catalase induction by nitrogenous substances and chelating compounds*

Addition to aeration medium		Growth Index (Klett Units)	Catalase activity (Unit/ml) Increase in 4 hrs	Inhibition (Per cent)	Reversal (Per cent)
None	...	76	248	...	...
$Fe^{+++}$	...	76	248	...	...
Cysteine	...	82	56	77.5	...
Cysteine + $Fe^{+++}$	...	79	138	44.4	33.1
Casein hydrolysate	...	104	128	48.4	...
Casein hydrolysate + $Fe^{+++}$	...	98	182	25.6	22.8
EDTA	...	76	88	64.5	...
EDTA + $Fe^{+++}$	...	76	168	32.2	32.3

The additions were: Cysteine 2 mg/ml, casein hydrolysate 5 mg/ml, EDTA 20 mg/ml and  $Fe^{+++}$  as 1 mg/ml of ferric ammonium citrate. Other details were as in Table I. Initial catalase activity was 24 units.



*Catalase synthesis during aerobic growth cycle:* From a study of the relationship of catalase activity to cell population changes during aerobic growth of the organism in the glucose-peptone-yeast extract medium, it was observed that the initial activity of catalase remained the same for about 4 hours although rapid increase in cell population occurred during this period (Fig. 5); activity increased subsequently and paralleled growth.

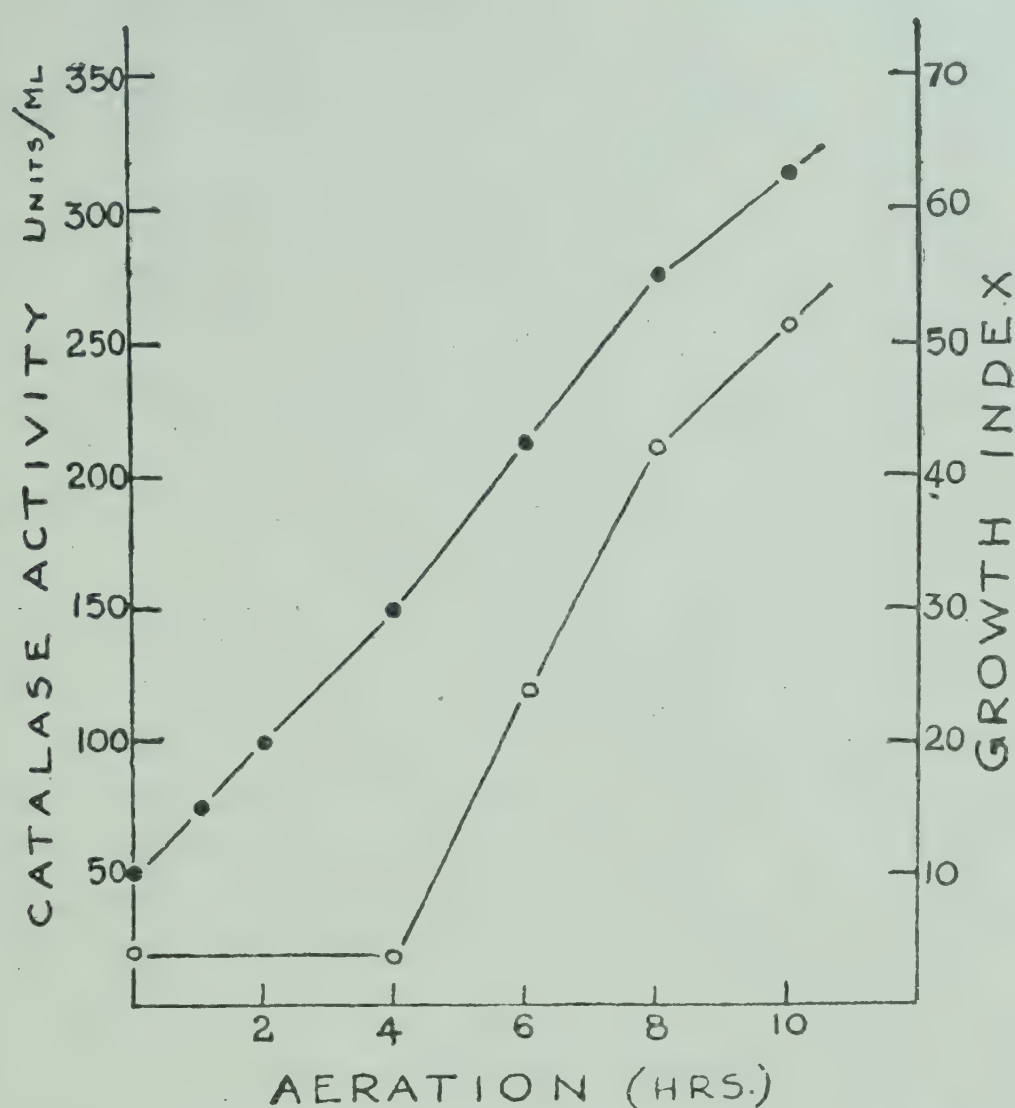


FIG. 5. Catalase synthesis during aerobic growth cycle. Cells were grown aerobically and analysed for catalase activity and growth periodically.  
●—● growth ; ○—○ catalase activity.

## Discussion

The nature of the appearance of the catalase activity may be either due to activation of an already existing zymogen or to *de novo* synthesis from the amino acid pool of the yeast cell. The need for an energy source such as glucose and for phosphate support the latter possibility. The appearance of catalase in *Rhodopseudomonas spheroides* has been demonstrated by Clayton<sup>12</sup> to be a *de novo* protein synthesis. The inhibitory effect of the purine and pyrimidine analogues further emphasises this.

The inhibition of catalase induction by 8-azaguanine may be either due to incorporation of the analogue into RNA fraction<sup>13-16</sup> or as recently suggested by Mandel and Markham<sup>17</sup>, the 8-azaguanine metabolite may act as an inhibitor of amino acid incorporation by antagonizing completely guanosine pyro-phosphate.

The activity of the RNA-hydrolysate in reversing the inhibitory effect of 8-azaguanine probably involves the synthesis of factors antagonized by 8-azaguanine or made non-functional by the incorporation of the analogue.



It may be noted that during catalase synthesis there is no net increase in RNA content. In fact, during phosphate starvation as well as in the inhibition by 8-azaguanine and its reversal by RNA-hydrolysate, there is some apparent degradation of cellular RNA on aeration. However, during aeration there is a steady increase in the incorporation of glycine-2-C<sup>14</sup> into RNA and protein, showing an active turnover of these constituents. It is thus likely that existing RNA is broken down during aeration and that the smaller units so formed are utilized for the build-up of new and specific RNA required for the adaptive synthesis of catalase.

The depression in catalase synthesis by added RNA preparation may be attributed to one or more of the following possibilities.

(i) The RNA not being just the specific one for catalase induction, may cause a general build-up of constitutive proteins in the system and thereby reduce the availability of the amino acids required for *de novo* synthesis of catalase.

(ii) There may be a carry-over of an inhibitory constituent together with ribonucleic acid.

(iii) The RNA or contaminating proteins in the preparation may act as source of nitrogen for a general build-up of non-specific proteins.

From the results (Table IV, Figs. 3 and 4) on the effects of an amino acid mixture and of casein hydrolysate, it would seem that utilizable nitrogenous compounds may promote general build-up of proteins in preference to the formation of the specific adaptive enzyme. Casein hydrolysate is also able to inhibit catalase formation even in the semi-adapted cells (Fig. 4). In all the cases studied, there is an increase in the cell population.

The above considerations are further substantiated by the observation that, with yeast cells growing under aerobic conditions favourable for rapid multiplication, increase in catalase activity of the culture is delayed upto a period of 4 hours. In fact, during this period there is an actual decline in catalase activity per cell.

However, the inhibitory effect may also be attributable to one or more compounds acting as anti-inducers. Thus, for example, compounds lowering the Redox potential of the system might be responsible for such an action through removal of hydrogen peroxide, the likely inducer. A compound chelating any essential metal ions might also bring about a similar effect. L-cysteine added singly could bring about a significant reduction in enzyme synthesis, whereas cystine does not affect enzyme formation appreciably. Again, EDTA inhibits catalase formation. These inhibitions could be reversed by addition of Fe<sup>+++</sup> which *per se* does not seem to have any appreciable influence. The inhibitory action of casein hydrolysate could obviously not be attributed to its cysteine content. Further, the iron salt could overcome the inhibition due to casein hydrolysate only to a small extent indicating that not all the inhibitory effect of the latter is due to the chelating action of its components. This is further borne out by the observation (Table V) that cysteine with casein hydrolysate in the aeration medium inhibits less than by itself.

It is thus likely that nutritional conditions favouring active multiplication of the cells allow little opportunity for the development of the adaptive enzyme.

## Summary

Observations are reported relating nucleic acid metabolism to induction of catalase activity on aeration of *Saccharomyces cerevisiae* cells grown under anaerobic conditions.



The involvement of ribonucleic acid (RNA) in catalase induction is demonstrable from studies on (i) the influence of phosphate starvation on catalase synthesis, (ii) inhibition of catalase induction with 8-azaguanine and pyrimidine antagonists and its reversal by RNA-hydrolysate and (iii) incorporation of glycine-2-C<sup>14</sup> into RNA and protein during catalase induction in spite of net reduction in total ribonucleic acid during this period. It is concluded that new and specific RNA is being synthesized at the expense of other RNA under conditions of forced synthesis of new protein. An RNA-rich extract of cells grown aerobically inhibits the appearance of the enzyme when added during aeration of the anaerobically grown cells. This effect could be due to non-RNA nitrogenous constituents as well. Thus casein hydrolysate, or an amino acid mixture or cysteine, also suppresses the enzyme synthesis. EDTA, a known chelating agent, is also inhibitory and the inhibition due to casein hydrolysate, cysteine and EDTA could be partially reversed by the addition of ferric salts.

It is suggested that catalase biosynthesis in yeast is only of secondary significance under nutritional conditions favourable for active multiplication.

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#### Discussion

Q. Why was RNA hydrolysate added?

A. It was added with a view to providing nucleotide, nucleoside and other precursors.

Q. What would be the concentration of free H<sub>2</sub>O<sub>2</sub> during the growth phase and what is its relation to catalase synthesis?

A. The H<sub>2</sub>O<sub>2</sub> concentration was not measured.

Q. Does decrease in catalase affect the viability of the cells?

A. No.



# EFFECT OF STREPTOMYCIN ON PROTEIN SYNTHESIS IN *ESCHERICHIA COLI*

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In connection with studies on the mode of action of streptomycin (SM) in *E. coli*, it was found that extensive excretion of nucleotides<sup>1,2</sup> and amino acids<sup>2</sup> takes place. In order to determine whether or not this excretion might result from, and/or contribute to, a defect in the incorporation of these metabolites, it seemed important to know the effect of SM on the formation of RNA, DNA, and protein.

SM has been shown to interfere with the formation of inducible enzymes<sup>3,4</sup> and to prevent incorporation of labelled amino acids in *Mycobacterium tuberculosis*<sup>5</sup>, but not in *E. coli*<sup>5</sup> and in *Staphylococcus aureus*<sup>6</sup>. Interference with incorporation of a labelled amino acid into protein has also been reported in experiments carried out with bacterial extracts<sup>7</sup>.

The evidence so far available does not provide a clear picture of the mode of action of streptomycin (SM). We have therefore studied the effect of SM on the kinetics of DNA, RNA and protein synthesis in *E. coli*, using both colorimetric estimation and determinations of the incorporation of labelled precursors. In addition, the formation of protein has been compared with the formation of an inducible enzyme,  $\beta$ -galactosidase. Finally, since it has been shown that depriving certain mutants of required amino acids or pyrimidine prevented the action of SM on viability and on metabolite excretion<sup>8</sup>, it seemed desirable to obtain additional evidence as to whether or not protein synthesis is essential for SM to exert these effects. Observations were, therefore, made on the influence of chloramphenicol (CA), a compound that specifically inhibits protein synthesis<sup>9</sup>.

## Experimental Materials and Methods

*Bacteriological.* The experiments were carried out (unless otherwise specified) with cultures of *E. coli* strain W growing exponentially with shaking at 37° in a mineral medium<sup>10</sup> with 0.5 per cent glucose. The cultures were prepared by transferring to 100 ml of medium a 2 ml inoculum of an overnight culture in the same medium. SM was added when the exponential growth had reached 0.2 mg dry weight, or  $4-5 \times 10^8$  cells per ml.

Viable counts were estimated by diluting in phosphate buffer and plating on tryptic digest agar. Colonies were counted after incubation of the plates for 18 hours at 37°.

Growth was measured turbidimetrically at 420 m $\mu$  in a Klett-Summerson photoelectric colorimeter. Dry weight of cells was estimated, after calibration, from the turbidity.



*Nucleic acid and protein measurement: RNA:* A 2 ml sample of the turbid culture was extracted twice with cold 5 per cent TCA. The residue was heated with 2 ml of 5 per cent TCA for 30 minutes at  $100^{\circ}$  and RNA was estimated in 1 ml of the supernate by the orcinol method<sup>11</sup> using sodium ribonucleate as standard.

*DNA:* 8 ml of the culture was extracted three times with cold 0.3 M perchloric acid, the residue was heated with 2 ml of 0.3 M perchloric acid at  $80-85^{\circ}$  for 30 minutes, and DNA was estimated in 1 ml of the extract by the indole method<sup>12</sup> using sodium deoxyribonucleate as the standard.

*Protein:* 1 ml of culture was acidified with TCA to a final concentration of 5 per cent. After centrifugation the residue was dissolved in 2 ml of 0.1 N sodium hydroxide solution and protein was estimated in 1 ml of the solution by the Folin's method<sup>13</sup> using bovine albumin as standard.

*Incorporation of amino acids, purines and pyrimidines:* As an independent index of nucleic acid and protein synthesis, the uptake and incorporation of radioactive amino acids, purine and pyrimidines, was studied. To an exponentially growing culture, the required concentration of SM was added, followed immediately by a solution of the amino acid to give a concentration of  $10\text{ }\mu\text{g/ml}$  or of the base to give a concentration of  $5\text{ }\mu\text{g/ml}$  and about 2000 c.p.m./ml. To measure total incorporation, 1 ml samples were withdrawn at various time intervals, cooled, diluted with a few ml of phosphate buffer, and filtered quickly through a membrane filter (Schleicher and Schuell 'coarse'). The filters were then washed with  $2 \times 5\text{ ml}$  of phosphate buffer and dried. To measure incorporation into protein or nucleic acid, cooled 1 ml samples were mixed with 1 ml of 10 per cent TCA, kept in the cold for about one hour, filtered through a membrane filter and washed with 5 ml of cold 5 per cent TCA and  $2 \times 5\text{ ml}$  of water. The filters were then dried and their radio-activity measured. The latter method of studying incorporation gave results comparable to those obtained by the more laborious procedures of extracting nucleic acid with hot 5 per cent TCA, followed by plating on plachets.

*Measurement of  $\beta$ -galactosidase synthesis:*  $\beta$ -galactosidase activity was measured according to the method of Cohn and Monod<sup>14</sup>, using o-nitrophenyl galactoside (obtained from Mann Biochemicals, U.S.A.)

## Results

*Effect of SM on RNA, DNA, and protein synthesis:* SM ( $60\text{ }\mu\text{g/ml}$ ) was added to an exponentially growing culture of *E. coli* (ca.  $3 \times 10^8$  cells/ml) in minimal medium and samples were taken at intervals and analysed as described above. Figure 1 shows that RNA synthesis continued at the same rate as that of untreated cells for almost 25-30 minutes, with a net increase in RNA of about 40 per cent. DNA synthesis continued at the normal rate for a bit longer (ca. 35-40 minutes) with an increase of about 55 per cent. Protein synthesis was stopped after about 15-20 minutes, with a net increase of only 10-15 per cent.

Since higher concentrations of SM are more rapidly bactericidal, the effect of a five-fold increase in SM was observed. As is seen in Figure 1, RNA and DNA syntheses were much the same as with  $60\text{ }\mu\text{g/ml}$  SM, but protein synthesis ceased almost immediately, and was limited to less than 5 per cent.



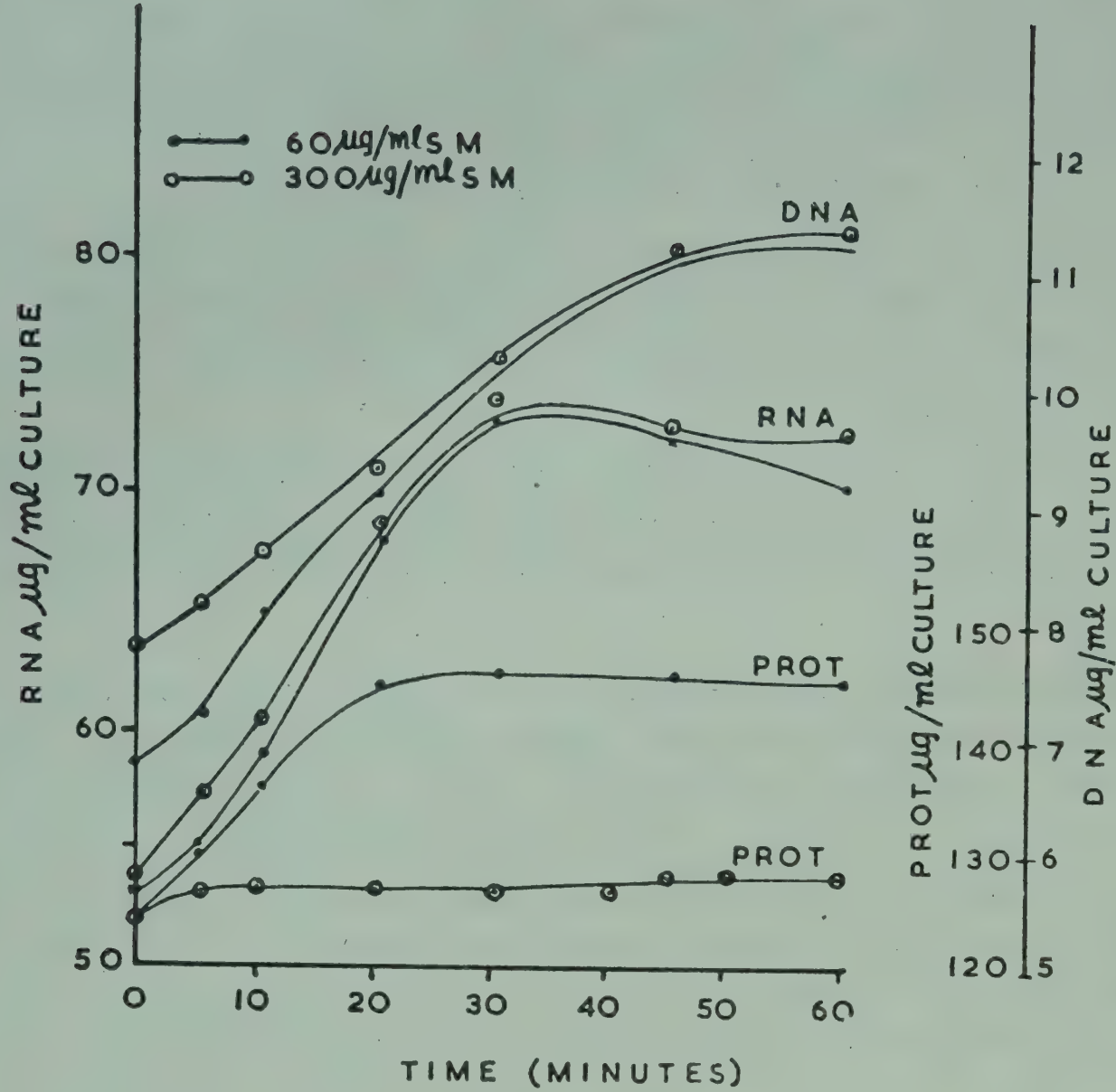


FIG. 1. RNA, DNA, and protein synthesis by an exponentially growing culture of *E. coli* when treated with 60 and 300 µg/ml SM

As an independent check on the synthesis of nucleic acids and protein in the presence of SM, the incorporation of C<sup>14</sup> labelled arginine and guanine was studied. The results obtained with arginine are presented in Table I. With 60 µg/ml SM, the incorporation stopped at about 15 minutes, almost simultaneously with the cessation of protein synthesis.

TABLE I  
*Incorporation of arginine-C<sup>14</sup> by E. coli cells treated with SM*

Control cells				Cells treated with 60 µg SM			Cells treated with 300 µg SM		
Time	O.D. 420	cpm/ml culture TCA insol.	Protein µg/ml	O.D. 420	cpm/ml culture TCA insol.	Protein µg/ml	O.D. 420	cpm/ml culture TCA insol.	Protein µg/ml
0'	103	10	148	110	10	160	92	8	138
5'	107	40	152	114	36	166	94	18	140
10'	114	64	158	118	64	174	94	18	142
20'	124	144	172	124	101	178	92	20	142
30'	133	228	190	124	110	179	92	18	142
45'	153	379	225	120	109	179	92	18	142
60'	179	540	288	116	113	178	93	18	142



With 300  $\mu\text{g/ml}$  SM, a greater inhibition was observed, and very little incorporation of arginine took place. Similar results were obtained with valine-1- $\text{C}^{14}$ .

The results on the uptake of guanine are presented in Table II. It will be seen that with both 60 and 300  $\mu\text{g/ml}$  SM, the rate of incorporation was the same as with untreated cells for about 30 minutes, after which it slowed down and completely stopped by about 45 minutes. These results are in conformity with those obtained by colorimetric estimation of the RNA and DNA of SM-treated cells. Cells preincubated with SM for as long as 30 mins (Table III) still continued to incorporate a fair amount of guanine 2- $\text{C}^{14}$  showing that fresh RNA synthesis was continuing even after 30 mins.

*Comparison of  $\beta$ -galactosidase with protein synthesis:* It appeared possible that the small amount of protein that is synthesised in the presence of SM is abnormal. As a convenient

TABLE II

*Incorporation of guanine-8- $\text{C}^{14}$  by E. coli cells treated with 60 and 300  $\mu\text{g/ml}$  SM\**

Control				Treated with 60 $\mu\text{g/ml}$ SM			Treated with 300 $\mu\text{g/ml}$ SM		
Time	O.D. 420	TCA insol. cpm/ml culture	RNA $\mu\text{g/ml}$ culture	O.D. 420	TCA insol. cpm/ml culture	RNA $\mu\text{g/ml}$ culture	O.D. 420	TCA insol. cpm/ml culture	RNA $\mu\text{g/ml}$ culture
0'	95	85	56	93	108	54	93	104	54
5'	99	395	57	97	418	55	96	403	56
10'	106	491	60	101	547	57	96	500	58
20'	117	520	70	110	585	67	96	550	66
30'	137	578	85	110	679	82	97	675	80
45'	162	628	104	110	650	83	97	580	82
60'	182	722	112	108	620	83	97	580	82

\* The medium contained 5  $\mu\text{g/ml}$  of guanine-8- $\text{C}^{14}$ , of 350 cpm/ $\mu\text{g}$  activity.

TABLE III

*Incorporation of guanine-8- $\text{C}^{14}$  by cells preincubated with 60  $\mu\text{g/ml}$  SM\**

Guanine and SM added together			Cells pretreated with SM for 30 mins before addition of guanine	
Time	O.D./490	TCA insol. cpm/ml culture	O.D./490	TCA insol. cpm/ml culture
0'	.294	10	.305	4
5'	.325	45	.295	45
10'	.352	124	.295	70
20'	.365	239	.300	185
30'	.376	400	.298	185
60'	.377	505	.268	172

\* The medium contained 5  $\mu\text{g/ml}$  guanine of 350 cpm/ $\mu\text{g}$  activity.



example of a specific protein, the formation of  $\beta$ -galactosidase, in minimal medium containing lactose (and no glucose) was studied. With  $60 \mu\text{g/ml}$  SM, synthesis of the enzyme stopped at the same time as that of protein and the brief period of synthesis did not permit the two phenomena to be dissected. However, with  $10 \mu\text{g/ml}$  SM (Fig. 2) it was seen that the protein synthesis continued at the rate of untreated cells for almost 60 mins, while the increment in enzyme level studied decreased after 20 mins. and stopped in about 30 mins.

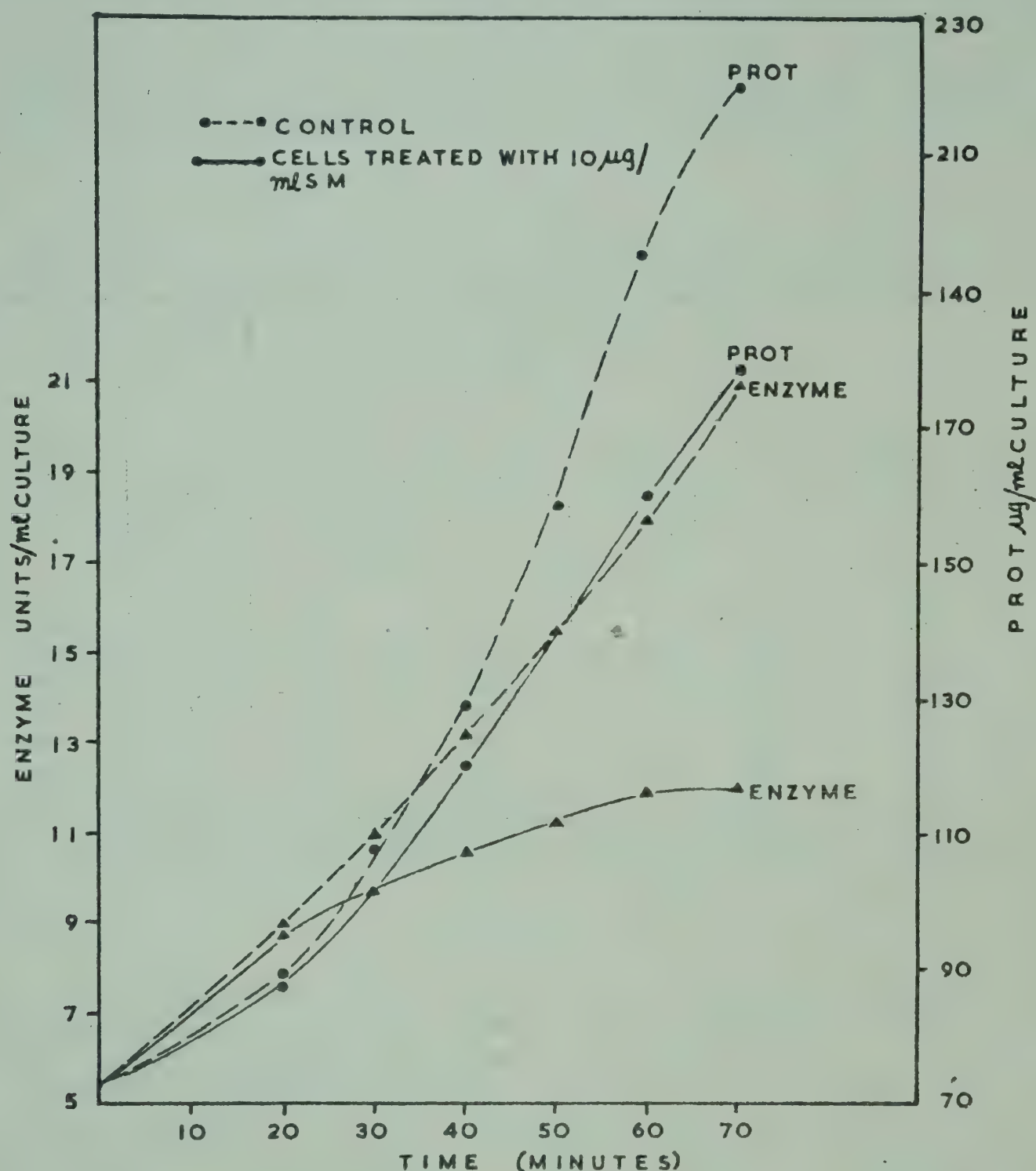


FIG. 2. Synthesis of  $\beta$ -galactosidase and proteins by *E. coli* cells treated with  $10 \mu\text{g/ml}$  SM.

*Protective effect of chloramphenicol on the metabolic effects of SM:* It has been reported that chloramphenicol (CA), a bacteriostatic agent, protects various bacterial species against the bactericidal action of SM<sup>15,16</sup>. As a background for the present metabolic studies, we established that when  $20 \mu\text{g/ml}$  of CA was added simultaneously with  $60 \mu\text{g/ml}$  of SM to an exponentially growing culture, the viable count remained constant for at least 2 hours as with the addition of CA alone, whereas SM alone caused rapid killing (Fig. 3) after 5 to 10 minutes. CA protection remained complete when its addition was delayed for as much as 5 mins after that of SM, but after a 10 mins delay there was no protection.



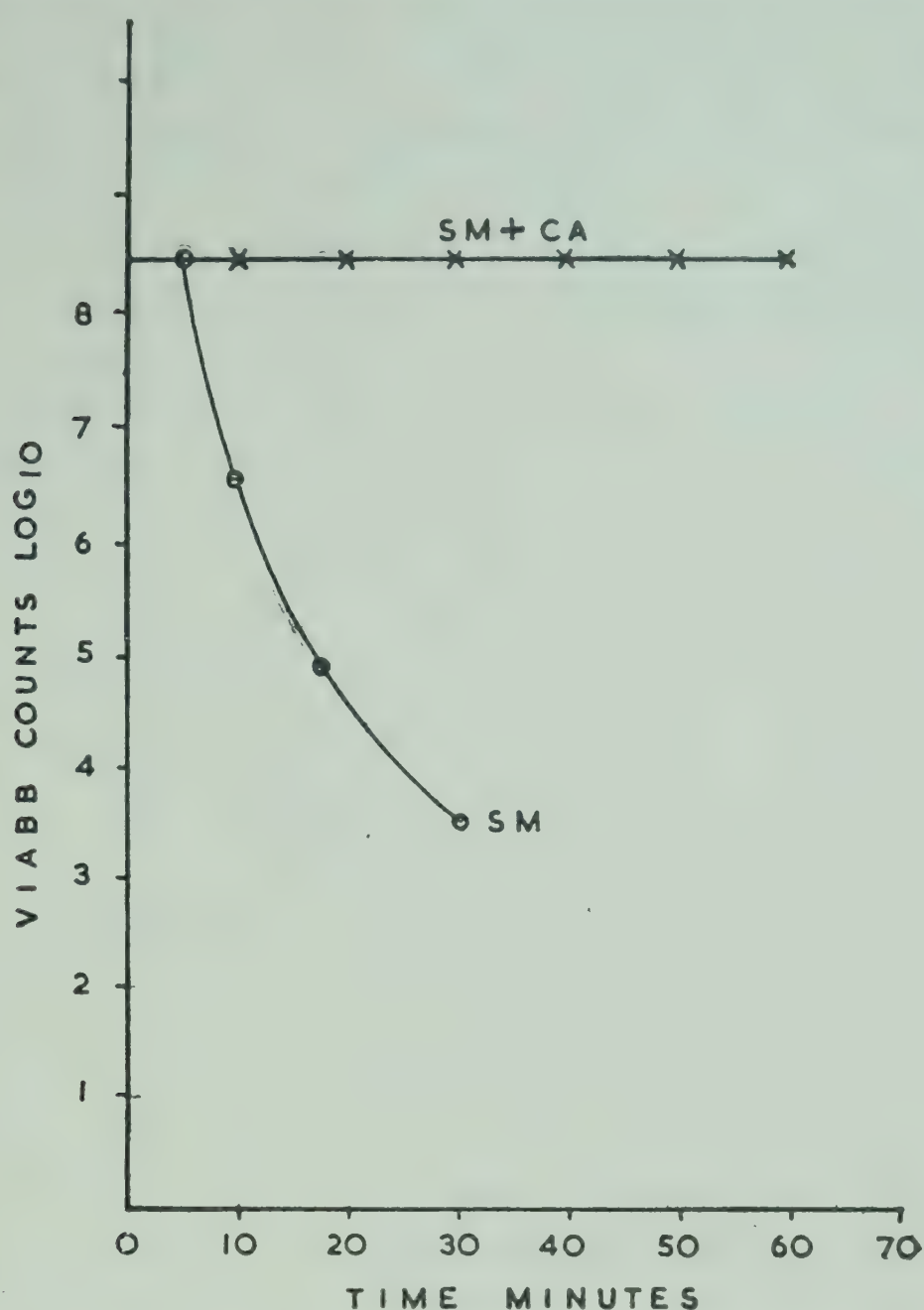


FIG. 3. Viable counts after treatment with SM alone and SM+CA.

The effects of SM and CA on nucleic acid and protein synthesis were next studied. As will be seen in Table IV, SM at this concentration allowed an initial continuation of the increase in turbidity of the growing culture which levelled off after 20 mins and then slowly

TABLE IV  
RNA, DNA and protein Composition of *E. coli* cells under SM and CA treatment

Treated with 60 $\mu$ g/ml SM					Treated with SM 60 $\mu$ g + CA 20 $\mu$ g/ml			
Time	O.D. 420	RNA $\mu$ g/ml culture	DNA $\mu$ g/ml culture	Prot. $\mu$ g/ml culture	O.D. 420	RNA $\mu$ g/ml culture	DNA $\mu$ g/ml culture	Prot. $\mu$ g/ml culture
0'	85	53	6.7	124	90	51	7.0	129
5'	88	55	7	130	90	54	7.5	130
10'	94	59	8	135	92	63	8.3	130
20'	102	68	9.5	144	94	72	9.4	128
30'	104	74	10.5	144	99	78	10.0	130
45'	98	74	11	144	103	83	10.5	130
60'	93	70	11	144	106	86	10.0	130



fell. In the presence of CA in addition to SM, the increase in turbidity was slower but lasted at least 90 mins. The effects of SM alone on DNA, RNA and protein synthesis presented in Table IV, are similar to those seen earlier in Fig. 1. In contrast, SM plus CA caused an abrupt cessation of protein synthesis and a more extensive synthesis of RNA (an increase of 70 per cent) than was seen with SM alone (an increase of 40 per cent).

All these effects of SM plus CA resemble those reported by others for the action of CA alone<sup>17</sup>. In addition, we have observed that in the presence of both SM and CA the marked excretion of 260 m $\mu$ -absorbing material, brought about by SM alone<sup>2</sup>, does not occur. It therefore appears that blockage of protein synthesis by CA completely prevents the characteristic metabolic effects of SM.

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# DIETARY PROTEIN QUALITY AND VITAMIN A UTILIZATION

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The level of protein in the diet has considerable influence on the storage of vitamin A by young rats<sup>1-3</sup> although no direct correlations have been found<sup>4,5</sup>. The studies of several investigators<sup>6-8</sup> reveal that dietary protein is in some way related to vitamin A utilization. The observations made in this laboratory by Esh, Bhattacharya and Som<sup>9</sup> showed that both the storage and utilization of vitamin A are affected in normal and protein depleted young adult rats when maintained on a low protein diet, particularly when the feeding period is long. In a recent preliminary report<sup>10</sup>, it has been shown that vitamin A storage was adversely affected when both sub-optimum level of imbalanced proteins (rice, wheat and Bengal gram pulse) were fed to vitamin A deficient young rats for long periods. Recheigl *et al.*,<sup>11</sup> however, observed in a comparative experiment lasting 3 weeks on normal animals that dietary amino acid imbalance has no effect on the liver storage of vitamin A.

This paper reports results on storage and utilization of vitamin A under different protein feeding conditions as well as on the levels of some liver enzymes at different intakes of vitamin A.

## Experimental

*Animals:* Thirty-day old weanling albino rats (38-45 g) bred and reared in the Institute's own colony were used in all these experiments.

*Diets:* The basal vitamin A-free diet contained vitamin-free casein, 18; salt mixture USP XIV, 4; refined *arachis* oil, 9; choline chloride, 0.2; vitamin mixture<sup>9</sup>, 1.5; and corn starch to make 100.

Casein, rice, wheat and Bengal gram pulse were used as the sources of dietary proteins for the preparation of experimental rations. In the case of rice and wheat, the protein level was increased by supplementing extra crude proteins isolated from these cereal sources. Wheat protein was extracted by gradual washing away the starchy materials from the prepared dough in cold running water. The protein was dried overnight under vacuum. The protein of rice was isolated by the dilute alkali extraction method<sup>12</sup>. The compositions of the experimental diets are given in Table I and all of them were free from vitamin A.

*Analytical methods:* Plasma (pooled samples) vitamin A was estimated according to Kimble<sup>13</sup>. Vitamin A was extracted from individual livers and bulked kidneys essentially by the method of Lewis *et al.*<sup>14</sup>. When a massive dose of vitamin A was fed, the extraction procedure followed was that of Horner and Morton<sup>15</sup>. Vitamin A was determined spectrophotometrically in isopropanol solvent using correction formulae of Cama, Collins and Morton<sup>16</sup>. Nitrogen in the diet was estimated by Kjeldhal method.



TABLE I  
Per cent composition of Experimental Diets

Components	Dietary groups (at 18% protein level)			
	Casein	Rice	Wheat	Pulse
Rice (including isolated rice protein) ...	...	82.5	...	...
Wheat (including isolated wheat protein) ...	...	...	64.3	...
Pulse (Bengal gram) ...	...	...	...	80.0
Vitamin-free casein ...	22.0	...	...	...
Corn starch ...	64.3	3.8	22.0	6.3
Salt mixture USP XIV ...	4.0	4.0	4.0	4.0
<i>Arachis</i> oil, refined ...	9.0	9.0	9.0	9.0
Choline chloride ...	0.2	0.2	0.2	0.2
Vitamin B complex <sup>9</sup> ...	1.0 ml	1.0 ml	1.0 ml	1.0 ml
Vitamin K, E and D mix <sup>9</sup> ...	0.5 „	0.5 „	1.5 „	0.5 „

Plasma and whole tissue homogenates of liver and kidney were used as enzyme sources for the estimation of cholinesterase<sup>17,18</sup>, xanthine oxidase<sup>19</sup> and succinic dehydrogenase<sup>20</sup>.

## Results

*Effect of short and long term feeding of different dietary proteins on the storage and distribution of vitamin A.* The animals were first made vitamin A-deficient on vitamin A-free diet, as evidenced by the onset of typical deficiency symptoms and cessation of growth. They were distributed into 5 matched groups having equal sex, weight and litters and placed in individual metal cages. *Ad libitum* feeding of experimental rations and distilled water was continued together with daily oral feeding of 45 I.U. of vitamin A palmitate. Weights were recorded twice weekly. 4 animals from each group were killed under light ether anaesthesia at the end of each experimental period and their plasma, liver and kidney were analysed for vitamin A. The results are shown in Table II. It is seen that the liver storage of vitamin A is not affected when diets having casein, pulse protein and no protein were fed for a period of two weeks only. This also indicates that vitamin A is absorbed in the system with equal efficiency irrespective of the quality or quantity of dietary protein. However, when the feeding of different protein diets was continued for 5 weeks, vitamin A was stored in greater amounts in the livers of rats fed an adequate protein like casein at optimum level. In the case of imbalanced proteins like those of rice, wheat and pulse, as well as low level of adequate protein, the animals stored less vitamin A in their livers. When compared with the casein 18 per cent fed group, these differences are highly significant ( $P = <0.01$  to  $<0.02$ ). Vitamin A in the plasma of rats in different groups also tends to show similar trend of variation. Little difference however, in the kidney levels has been observed. Ingestion of 60 I.U. of vitamin A daily to normal weanling rats for one week when fed casein, protein free and pulse protein diets also showed no variation in the liver storage of vitamin A (Table III). This is possibly due to the short period of feeding.



TABLE II

*Effect of feeding different proteins to vitamin A-deficient weanling rats on the storage and distribution of vitamin A when dosed with 45 I.U. of vitamin A daily*  
(Average of 4 rats in each group)

Sources and level of protein	Weight gain, g	Plasma vit. A I.U./100 ml	Liver wt. g	Liver vit. A I.U.	Kidney wt. g	Kidney vit. A I.U.
<i>At the end of 2 weeks</i>						
Casein (18% protein)	21±1.4*	49.80	3.81	103±13.4	N.D.	N.D.
Protein deficient	-16±0	58.10	3.67	123±7.8	N.D.	N.D.
Pulse (18% protein)	13.5±2.1	44.90	3.52	111±7.1	N.D.	N.D.
<i>At the end of 5 weeks</i>						
Casein (18% protein)	71±8.7	94.6	4.43	268±39.7	0.99	3.15
Casein (6% protein)	11±3.2	65.8	3.56	97±17.7	0.89	5.8
Rice (18% protein)	50±3.7	75.8	4.56	186±30.9	0.99	4.5
Wheat (18% protein)	47±6.1	71.4	4.76	162±19.4	0.98	4.5
Pulse (18% protein)	46±2.4	73.0	4.36	140±34.8	0.93	4.0

\* Mean±standard error of the mean.

N.D.=Not Determined.

TABLE III

*Storage of vitamin A in the liver of normal weanling rats (4 in each group), after feeding different protein diets and 60 I.U. of vitamin A daily for one week*

Groups	Wt. gain g	Liver weight g	Liver vitamin A I.U.
Casein (18% protein) ...	9.5	2.91	104.6
Protein deficient ...	-8.0	1.85	99.9
Pulse (18% protein) ...	5.5	2.89	108.4

*Effect of different sources of dietary proteins on the rate of depletion of vitamin A:* Normal weanling rats were fed stock laboratory diets for 7 days and were fed orally with 1800 I.U. of vitamin A palmitate daily for 3 days. After allowing a day's rest, the animals were distributed equally into different groups according to body weight, sex and litters, and were housed in individual metal cages. Groups of these animals were given experimental rations with distilled water *ad libitum*. Weights were recorded twice weekly. Similar collection of blood, liver and kidney was made at the end of each experimental period and the vitamin A content of these organs was analysed. It is evident from Table IV that all the animals retained more or less the same amount of vitamin A in their livers when fed different protein diets for 2 weeks. Though the protein deficient group is found to retain slightly more vitamin A, the difference is very insignificant when calculated statistically ( $P \geq 0.3$ ). A different picture is, however, observed at the end of 5 weeks, where imbalanced protein feeding conditions were found to reduce significantly ( $P = < 0.01$  to  $< 0.02$ ) the liver storage of vitamin A, pulse protein being most adversely affected. There is no significant variation in either the kidney storage or plasma level of vitamin A.



TABLE IV

*Depletion of vitamin A under the influence of different sources of dietary protein. Massive dose of vitamin A was fed before giving experimental rations. (Average of 4 rats in each group)*

Sources and level of dietary protein		Weight gain g	Plasma vit. A I.U./100 ml	Liver weight g	Liver vit. A I.U.	Kidney weight g	Kidney vit. A I.U.
<i>At the end of 2 weeks</i>							
Casein (18% protein)	...	32 ± 2.8	39.1	3.19	3363 ± 221	0.72	12.3
Rice ( " " )	...	25.5 ± 2.1	43.7	3.13	3502 ± 202	0.58	11.4
Wheat ( " " )	...	21 ± 4.2	47.2	3.21	3520 ± 78	0.67	9.3
Pulse ( " " )	...	16.5 ± 2.1	41.1	3.21	3538 ± 120	0.74	10.4
Protein deficient	...	-16.5 ± 2.1	46.1	1.45	3774 ± 85	0.37	12.9
<i>At the end of 5 weeks</i>							
Casein (18% protein)	...	77 ± 2.8	47.6	4.39	2321 ± 133	0.84	10.9
Rice ( " " )	...	43 ± 2.8	68.1	4.48	1744 ± 145	0.76	11.9
Wheat ( " " )	...	40 ± 2.8	62.6	4.04	1577 ± 109	0.75	16.3
Pulse ( " " )	...	31 ± 1.4	38.8	3.75	1128 ± 87	0.65	13.3

*Effect of different intakes of vitamin A on certain enzyme activities of the system:* Tissue enzymes are known to be affected in dietary protein inadequacy. It was of interest to study the levels of these enzymes at different intakes of vitamin A. Groups of weanling rats deficient in vitamin A and a control group were fed basal ration simultaneously with different levels of vitamin A for a period of 5 weeks. At the end of the experimental period, the animals were killed by cardiac puncture under light ether anaesthesia. Blood was collected in an oxalated syringe and centrifuged immediately to obtain plasma. Livers and kidneys were quickly removed, chilled in ice, washed to remove adhering blood and fatty tissues, blotted on a filter paper and weighed. Weighed portions of tissues were homogenized in appropriate buffers in a Potter-Elvehjem homogenizer, strained through gauze and used for enzyme estimation.

Compared to the normals, vitamin A deficient animals have lower activity of succinic dehydrogenase both in the liver and kidney (Table V). While cholinesterase activity tends to decrease in vitamin A-deficient state, xanthine oxidase activity seems to remain unaltered. Succinic dehydrogenase activity tended to increase with increasing intake of vitamin A up to 60 I.U. and then it levelled off. Level of vitamin A seems to have no influence on xanthine oxidase activity in the liver, and optimum level of cholinesterase activity both in the liver and plasma was observed at the daily intake of 60 I.U. of vitamin A.

## Discussion

Our observations suggest that, under conditions of inadequate protein feeding, initial storage of vitamin A does not suffer but continued intake of dietary proteins having lower biological value as well as sub-optimum level of biologically adequate protein seem to affect adversely the normal storage and utilization of vitamin A. The slightly higher liver storage of vitamin A reported on feeding protein deficient diet<sup>5,9</sup>, low protein diet<sup>9</sup> or a diet with imbalanced protein<sup>11</sup> was on the basis of observations made during an



TABLE V

*Enzyme activities of plasma, liver and kidney in control and vitamin A-deficient rats and deficient rats when repleted at different levels of vitamin A for 5 weeks*  
(Average of 4 rats in each group)

	Normal Control	Vitamin A deficient	Repleted groups		
			I	II	III
Daily vitamin A supplement, I.U.	45	Nil	20	60	100
Weight gain, g ...	56 ± 5.4	31 ± 4.6	62 ± 1.7	63 ± 3.8	67 ± 2.8
Liver weight, g ...	4.56	3.29	5.01	5.78	5.20
Cholinesterase,* liver ...	63.9 ± 3.66	52.5 ± 7.49	72.8 ± 2.50	78.8 ± 8.51	70.7 ± 4.51
„ plasma ...	37.9 ± 1.72	33.5 ± 3.07	45.5 ± 7.80	52.7 ± 6.60	34.8 ± 2.47
Succinic dehydrogenase,† liver	5.29 ± 0.75	2.08 ± 0.16	4.91 ± 0.61	6.53 ± 0.18	6.55 ± 0.09
„ „ kidney	7.08 ± 1.05	4.15 ± 0.28	5.13 ± 0.18	6.66 ± 0.93	6.59 ± 0.39
Xanthine oxidase,‡ liver ...	6.17 ± 0.40	7.60 ± 0.70	5.58 ± 0.33	4.19 ± 0.50	4.66 ± 1.83

\*  $\mu$ M acetyl choline/hour/gm liver, or/ml plasma.

† Change in optical density at 400 m $\mu$ /min/gm fresh tissue at room temperature.

‡  $\mu$ M xanthine/hour/gm liver.

experimental period of only 2-3 weeks. The slightly higher liver storage of vitamin A associated with 2 weeks protein deficiency is also observed in the present investigation (Table IV). Statistically, however, this increase is very insignificant.

The lowering of absorbed and stored vitamin A level in the liver after feeding either sub-optimum protein diets or diets containing inferior quality of protein for long periods, indicate that either vitamin A is diverted to some other tissues, or vitamin A is destroyed in the blood, or vitamin A is utilized abnormally by the system. The negligible difference in the vitamin A content of kidney, the next possible storage depot, rules out the first possibility. Vitamin A may have been destroyed in the blood after mobilisation from the liver, due to the altered activities of enzyme systems thus reducing the liver storage. But no significantly apparent relationship between vitamin A intake and levels of some of the enzymes in the liver and or plasma, thus far studied, is observed in the present investigation. Several authors<sup>21-23</sup> have, however, shown that both carotene and vitamin A enhanced the activity of succinic dehydrogenase. It has been observed in these studies that succinic dehydrogenase activity is significantly reduced in vitamin A deficiency and optimum enzyme level has been observed when the daily dose of vitamin was 60 I.U. To what extent the levels of cholinesterase and xanthine oxidase are influenced by the daily intake of vitamin A is difficult to assess from this preliminary study; further detailed work is being continued to establish their relationships.



## Summary

Results of the present investigation tend to show that, though inadequate or sub-optimum protein feeding has no ill effect on the utilization or on liver storage of vitamin A for a short period, continued intake of imbalanced vegetable proteins as available from cereals and pulses, or even a low intake of casein, will adversely affect the normal storage and utilization of vitamin A either through destruction or through improper utilization. The reverse phenomenon is observed when adequate amount of protein in the form of casein is ingested by animals over a period of 5 weeks. Further, limited data, collected in this preliminary study, suggest a possible relationship between the daily vitamin A intake and levels of some enzymes in the liver and/or plasma most susceptible to dietary protein.

## Acknowledgment

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## Discussion

- Q. In protein malnutrition in humans the plasma vitamin A levels are low and improve on protein rehabilitation. Could this be ascribed to better storage of the vitamin in the liver?



- A. Possibly. The increase in plasma protein fractions—the lipoproteins which specifically bind the vitamin A—may also be responsible for the enhanced levels observed.
- Q. Maximal amount of vitamin A in the liver is observed when the protein level of the diet is 12% and higher levels lower the liver content of the vitamin. How is this to be understood?
- A. There may be increased requirement of and transference to other tissues. It is well-known that as growth increases there is lower storage of vitamin A in the liver.
- Q. In protein malnutrition there is reported to be a vitamin A destruction—factor in the erythrocytes which decreases during treatment through protein supplementation. Is there evidence of such a factor in the liver?
- A. No such factors seem to have been reported so far.



# HEMATOPOIESIS AND NITROGEN METABOLISM IN THE RIBOFLAVIN DEFICIENT RAT

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Anemia or impaired hemoglobin production has been reported in several species of animals receiving a riboflavin deficient diet<sup>1-5</sup>. A possible role of riboflavin in hematopoiesis is, however, not known. The present study was undertaken to ascertain its influence in hematopoiesis and protein anabolism in tissues, such as the liver and kidney. Many studies have indicated a relationship between riboflavin and protein metabolism<sup>6-12</sup>. Perhaps one of the most important findings is a direct relationship between the amount of protein in the diet of rats and the concentration of riboflavin in the liver<sup>9</sup>. There is also some evidence that not only the synthesis or elaboration of prosthetic groups of certain flavin enzymes, but also of their protein components depends upon an adequate intake of riboflavin<sup>13-17</sup>.

## Experimental

Albino rats of both sexes with initial weights ranging from 67 to 105 g were used for experiments on hematopoiesis and nitrogen metabolism. They were housed individually in raised screen bottom cages. Water was supplied *ad libitum*. The percentage composition of the basal diet was as follows: vitamin test casein 16, sucrose 69, cellulose flour 4, corn oil 7 and salt mixture 4. To every 100 g of this mixture were added: cod liver oil concentrate 0.2 mg,  $\alpha$ -tocopherol acetate 3 mg, 2-methylnapthoquinone 0.2 mg, choline chloride 100 mg and inositol 50 mg. Changes in the protein content of the diets were compensated for by altering the content of sucrose. Water soluble vitamins were given to each rat daily by subcutaneous injection in 1 ml of a saline solution which contained the following: thiamin chloride 12.5  $\mu$ g, pyridoxine hydrochloride 10  $\mu$ g, calcium pantothenate 100  $\mu$ g, nicotinic acid 20  $\mu$ g, biotin 2  $\mu$ g, folic acid 2  $\mu$ g and vitamin B<sub>12</sub> 0.01  $\mu$ g. In each experiment, the rats were divided into three groups of the same initial average weight, and those of the two groups, the food controls and the weight controls, were given 25  $\mu$ g of riboflavin in 1 ml saline per day with the other water soluble vitamins. The same amount of food was consumed by each rat of the first control group (food control) daily, this being governed by the amount voluntarily consumed by those deprived of riboflavin. The weight control animals received the basal diet in daily quantities just sufficient to maintain weight equal to that in the deprived group.

*Red blood cells and hemoglobin (Experiment 1):* Food control, weight control and riboflavin deprived rats, twenty in each group, were maintained on the experimental regimen for 65 days. At the end of this time, the three groups had gained in weight an average of 70, 44 and 42 per cent respectively. Each day for the next 12 days, the blood



of some animals of each group was examined. It was obtained by cutting the end off the tail, which had been warmed in water, and was taken directly into pipettes. Hemoglobin and hematocrit were determined by the method of Collier<sup>18</sup> and Van Allen<sup>19</sup> respectively. Red cell counts were done by the standard methods, the hemocytometer pipettes used being within the tolerance required by the U.S. Bureau of Standards. Mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration were calculated.

In the succeeding experiments hemoglobin was measured by the same method as in experiment 1.

*Hemorrhage (Experiment 2):* Food control, weight control and riboflavin deprived rats, eight in each group, had gained in weight an average of 85, 47 and 50 per cent respectively after 78 days on the experimental regimen. At this time, each rat was subjected to hemorrhage from the cut tail on each of 2 alternate days, the techniques used being the same as those of Kornberg *et al*<sup>20,21</sup>. At each hemorrhage, blood equivalent to 2 per cent of the body weight was removed, following which 5 c.c. of saline were given intraperitoneally. Just prior to each hemorrhage and at intervals thereafter for 26 days, hemoglobin values were obtained on caudal blood.

*Cobalt administration (Experiment 3):* Food control, weight control and riboflavin deprived rats, twelve in each group, gained in weight an average of 83, 57 and 58 per cent respectively, in 84 days on the experimental regimen. Beginning at this time each rat was given a daily intraperitoneal injection of 0.3 mg cobalt in 1 ml of a saline solution of cobalt chloride. Prior to the first injection and thereafter at intervals for 49 days hemoglobin determinations were made on caudal blood.

*Regeneration of liver nitrogen after protein starvation and hemorrhage (Experiment 4):* Food control, weight control and riboflavin deprived rats, seventeen to twenty in each group, gained in weight an average of 44, 24 and 16 per cent respectively, in 45 days on the experimental regimen. Eight animals from each group were killed, and hemoglobin<sup>18</sup> plasma protein<sup>22</sup> and liver nitrogen<sup>23,24</sup> values were obtained. The remainder for the next 20 days were maintained on a protein-free diet and then were subjected to a single hemorrhage, blood equivalent to 2 per cent of the body weight being removed. The next day four animals from each group were killed for the determination of hemoglobin, plasma protein and liver nitrogen. The remainder (seven to eight in each group) were maintained on a 40 per cent protein diet for 3 days and then killed to obtain the same data.

*Liver nitrogen in riboflavin deficiency (Experiment 5):* This experiment was designed to obtain more information on the content of liver nitrogen in riboflavin deficiency, using a large number of animals. Food control, weight control and riboflavin deprived rats,<sup>19-20</sup> in each group, gained in weight an average of 65, 57 and 55 per cent respectively in 57 days on the experimental regimen. The animals were killed and plasma protein and liver nitrogen values were determined.

*The content of nitrogen, fat and water in the liver and of nitrogen in the kidneys in riboflavin deficiency (Experiment 6):* In view of some of the results of the previous experiments, an experiment was done in which information was obtained on the fat, nitrogen and water contents in liver tissue of rats in riboflavin deficiency. Nitrogen levels in the



kidneys were also obtained. In this experiment food control, weight control and riboflavin deprived rats,<sup>18-20</sup> in each group, were used. They gained in weight an average of 85, 47 and 44 per cent respectively in 51 days of riboflavin deficiency.

## Results

The observations recorded in Table I indicate no significant alteration of the hematological picture in riboflavin deficiency, or in inanition, as shown by the weight controls.

TABLE I. *Riboflavin deficiency in the rat: Red blood cells and hemoglobin*

	Red cells Millions mm. <sup>3</sup>	Haematocrit Vol. %	Hemoglo- bin g./100 ml.	M. c. v. $\mu^3$	M. c. Hb $\mu\mu\text{g.}$	M. c. Hb con. %
<b>A</b>	Ranges with Averages and S.D.					
Pair fed: ...	7.15-9.54	38-48	12.7-15.8	47-60	16-20	30-35
Food controls (20 Rats)	$8.13 \pm 0.63$	$44 \pm 2.32$	$14.7 \pm 0.75$	$55 \pm 3.5$	$18 \pm 1.4$	$33 \pm 1.2$
<b>B</b>						
Inanition: ...	7.44-9.12	41-49	13.2-16.0	49-65	16-19	30-35
Wt. controls (20 Rats) ...	$8.28 \pm 0.45$	$44 \pm 2.02$	$14.7 \pm 0.73$	$53 \pm 3.4$	$18 \pm 1.1$	$33 \pm 1.5$
<b>C</b>						
Riboflavin ...	7.33-9.76	37-47	13.1-15.6	42-63	15-20	31-37
Deficient (20 Rats) ...	$8.50 \pm 0.68$	$43 \pm 2.44$	$14.5 \pm 0.64$	$51 \pm 5.2$	$17 \pm 1.5$	$34 \pm 1.4$
<b>P Values</b>						
A-B	<.4	<.8	<.8	<.3	<.3	<.9
A-C	<.1	<.2	<.3	<.02	<.05	<.3
B-C	<.3	<.2	<.5	<.1	<.2	<.3

Figure 1 shows that deprivation of riboflavin lowered the capacity for blood regeneration, the evidence being a difference from controls in the rate of fall and of recovery in the hemoglobin after hemorrhage. Two days after the last hemorrhage, the average hemoglobin values of food control, weight control and riboflavin deprived groups were 41, 42 and 45 per cent below their respective initial averages. 18 days later, the hemoglobin values of the three groups had risen to 9, 20 and 25 per cent below the initial average values and the values tended to remain at that level for the following 6 days during which they were studied.

Figure 2 shows that the polycythemic response to cobalt occurred in both groups of control animals to the extent of a rise in hemoglobin over the initial values to 12 and 12.2 per cent respectively, whereas in riboflavin deficiency the values differed to an extent of only 6.4 per cent.

The results presented in Table II show that rats with riboflavin deficiency had essentially the same concentration of nitrogen in the liver as did those of both control groups, but the livers were larger in proportion to body weight. Consequently, the ratio of liver nitrogen to body weight was greater. After a 20-day period of protein starvation and a



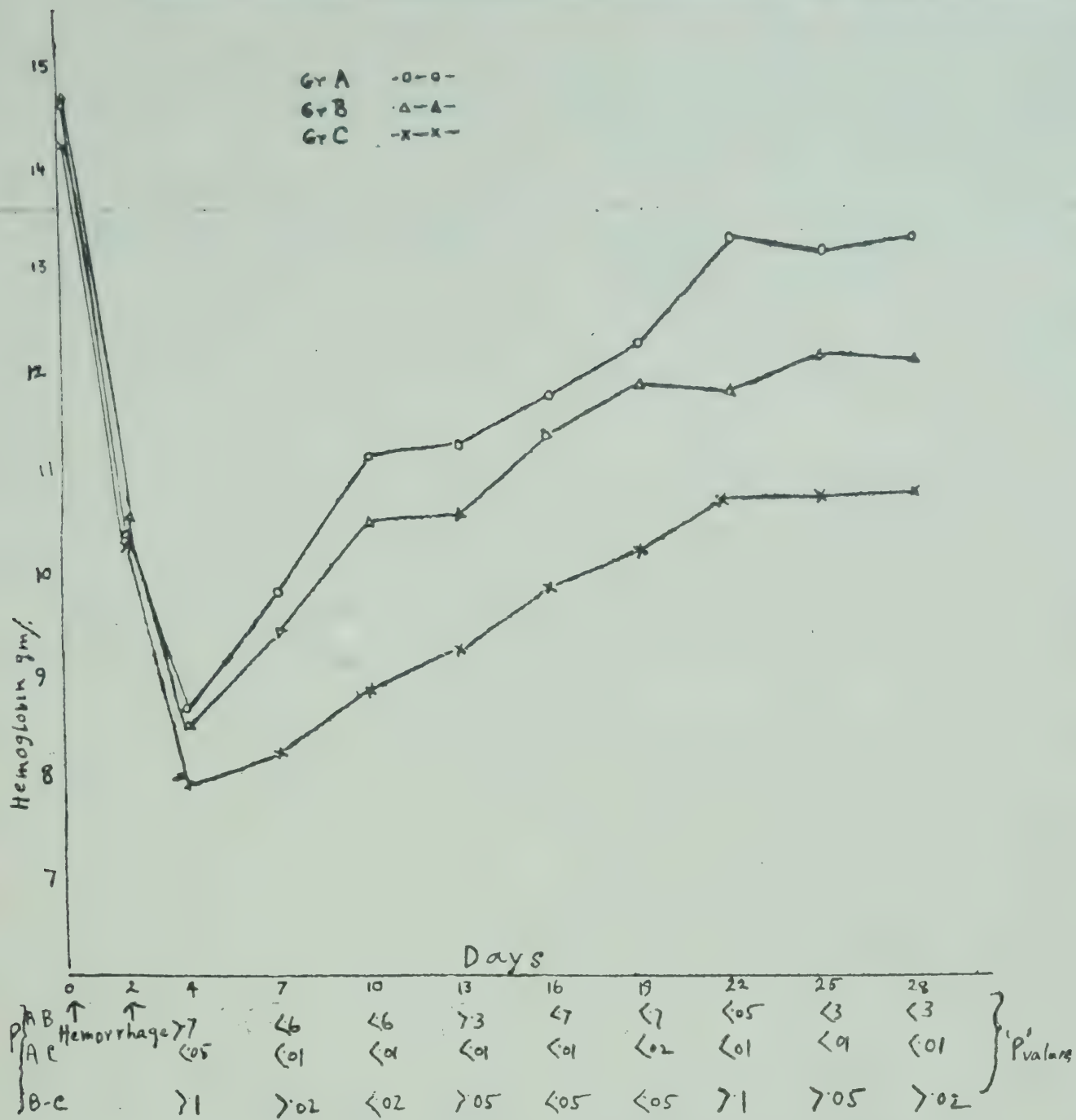
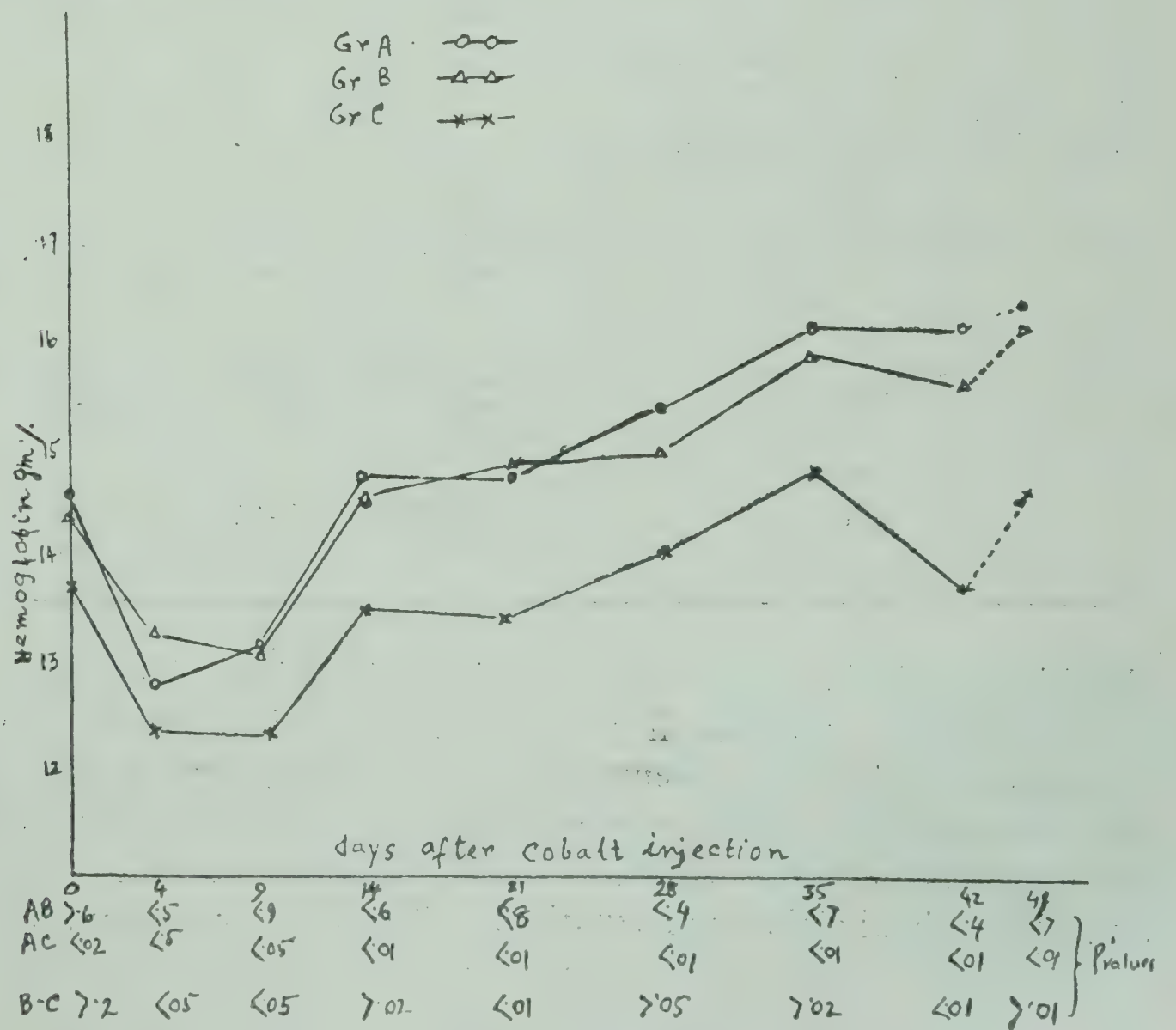
Fig. 1 Regeneration of hemoglobin after hemorrhageFig. 2. Cobalt polycythemia in riboflavin deficiency



TABLE II

*Riboflavin deficiency in the rat: the regeneration of liver and blood proteins after protein starvation and hemorrhage*

Regimen	Hemo- globin g/100 ml	Plasma protein g/100 ml	g N/100 g Liver	mg. Liver N/100 g Body wt.	Liver wt. Body wt. × 100
Ranges and Averages					
After 45 days on 16% protein diet					
A (Food controls) (8 Rats)	13.8-15.9 14.5	3.6-4.4 3.9	3.0-3.8 3.4	97.4-118.7 103.5	2.6-3.6 3.1
B (Weight controls) (8 Rats)	12.5-15.1 13.7	3.0-4.0 3.4	3.1-3.7 3.5	88.1-109.9 99.7	2.4-3.4 2.9
C (Riboflavin deficient) (8 Rats)	11.4-14.9 13.4	2.3-4.3 3.6	3.0-3.5 3.3	110.5-150.0 136.0	3.4-4.8 4.2
After 20 days on protein-free diet and next day after hemorrhage					
A (4 Rats)	7.4-8.9 8.1	2.2-3.3 2.8	2.4-2.7 2.6	80.6-95.4 87.0	3.3-3.6 3.4
B (4 Rats)	5.8-6.9 6.5	2.0-2.6 2.2	2.3-2.7 2.5	97.6-107.1 102.7	3.7-4.7 4.2
C (4 Rats)	5.3-6.6 6.0	2.0-2.7 2.3	2.4-2.6 2.5	88.1-113.0 103.3	3.5-4.8 4.1
After 3 days on 40% protein diet and third day after hemorrhage					
A (8 Rats)	9.4-10.7 9.9	4.4-5.4 4.8	3.3-3.9 3.4	104.4-136.4 127.3	3.5-4.0 3.8
B (7 Rats)	7.9-10.4 9.1	4.2-4.8 4.5	2.9-3.3 3.1	111.6-158.4 128.1	3.5-4.9 4.1
C (8 Rats)	8.4-10.5 9.5	4.3-4.7 4.5	3.0-3.6 3.2	137.2-180.5 153.9	4.0-5.9 4.7

blood loss equivalent to 2 per cent of the body weight, the concentration of liver nitrogen fell to the same extent in all groups. During a subsequent short period of feeding on a high protein diet, the concentration of liver nitrogen rose to the same extent in all groups, but in the riboflavin deficient animals the ratio of liver to body weight and consequently of liver nitrogen to body weight increased to a greater extent. Values for hemoglobin and plasma proteins in the same experiment did not show any appreciable differences.



The results in Table III present evidence that in riboflavin deficient animals, the ratio of liver to body weight and consequently of liver nitrogen to body weight is greater. This supports the data in Table II, which shows the results of an experiment on a smaller number of animals.

The results in Table IV show further that the ratios of liver to body weight and of kidney to body weight were greater in another set of riboflavin deficient animals. The results presented in Table V, however, do not show the increase in the ratio of liver nitrogen to body weight in riboflavin deficiency, but the deficient animals maintained their liver nitrogen concentrations at the same levels as the food controls, whereas the inanition controls showed decline. Results presented in this table further show that the ratio of dry liver substance to body weight was greater in the deficient group, which indicates an actual increase of the liver tissue substance in riboflavin deficiency.

The results presented in Table VI show that in riboflavin deficient animals, the kidneys show no differences in regard to either the concentration of nitrogen or the ratio of kidney nitrogen to body weight.

Discussion

The results reported here indicate that 65 days of riboflavin deficiency does not produce in the rat any significant changes in the hematological picture. The results are in agreement with those of Carpenter and Kodiceck<sup>25</sup>. The hematopoietic system is, however, impaired in riboflavin deficiency. This is evident from the results showing impairment of hemoglobin formation under conditions of repeated hemorrhage and cobalt intoxication, even when the effects of inanition and impaired growth are taken into account. An experiment similar to those reported here has shown that riboflavin is a hematopoietic factor for dogs<sup>1,2</sup>.

TABLE III  
Riboflavin deficiency in the rat: plasma protein and liver nitrogen

Regimen	Body weights, g		Plasma protein g/100 ml	Liver wt. g	Total Liver N mg	g.N/100g Liver	Mg Liver N/100g. Body wt.	Liver wt./Body wt. × 100
	Initial	Final						
<b>A</b>	Ranges and Averages							
Food controls ... (19 Rats) ...	82-104 95	141-177 157	3.4-4.3 3.8	4.8-5.6 5.2	166.5-191.9 176.9	3.2-3.7 3.4	103.3-120.4 113.1	2.9-3.7 3.3
<b>B</b>								
Weight controls ... (20 Rats) ...	82-105 95	137-167 149	3.5-4.7 3.9	4.2-5.2 4.6	151.1-181.5 163.9	3.2-3.7 3.5	103.3-117.9 110.0	2.7-3.5 3.1
<b>C</b>								
Riboflavin deficient (19 Rats) ...	83-103 95	119-167 147	2.8-4.3 3.6	4.8-7.1 5.6	167.9-227.6 195.9	2.9-4.4 3.5	110.5-159.1 133.6	3.2-4.9 3.8



TABLE IV. *Flavin deficiency in the rat: Hemoglobin, plasma protein and liver and kidney weights*

		Body weights, g.		Liver wt. g	Wt. of kidneys, g.	Liver wt./body wt. ×100	Kidney wt./body wt. ×100	Hemoglobin, g./100 ml.	Plasma protein g./100ml.
		Initial	Final						
<b>A</b>		Ranges and Averages							
(Food controls)	...	67-90	132-158	4.1-5.1	0.9-1.4	2.8-4.4	0.65-0.99	13.6-16.5	3.3-4.5
(18 Rats)	...	80	148	4.5	1.2	3.1	0.78	15.5	3.8
<b>B</b>									
(Weight controls)	...	72-92	99-126	2.7-3.7	0.8-1.1	2.5-3.0	0.70-1.02	14.1-16.7	3.3-4.5
(20 Rats)	...	80	116	3.3	0.9	2.8	0.83	15.0	3.7
<b>C</b>									
(Riboflavin deficient)	...	65-94	94-143	3.3-4.8	0.8-1.1	2.8-3.5	0.72-0.98	13.6-16.1	3.0-4.1
(20 Rats)	...	79	114	4.1	1.0	3.5	0.86	15.0	3.6

TABLE V. *Riboflavin deficiency in the rat: the levels of nitrogen, fat and water in the liver*

Regimen	g N/100 g Liver	mg Liver N/100 g Body wt.	Total liver N mg	mg Fat/g Liver	mg. Liver fat/100g Body wt.	Total liver fat, g	g Water/g Liver	g Dry Liver 100 g Body wt.
Ranges and Averages								
<b>A</b>								
Food controls	2.5-3.7	80.7-117.3	122.8-180.6	2.7-15.3	8.1-45.0	13.2-78.4	0.68-0.72	0.809-1.032
(18 Rats)	3.1	94.9	140.3	9.8	29.8	43.9	0.70	0.912
<b>B</b>								
Weight controls	2.5-3.6	70.3-105.7	73.8-118.2	3.2-25.9	9.5-78.4	10.5-95.7	0.68-0.73	0.744-0.928
(20 Rats)	3.1	87.7	101.17	9.7	26.0	30.5	0.71	0.817
<b>C</b>								
Riboflavin deficient	2.5-3.7	70.3-126.3	89.8-157.0	1.4-18.7	4.2-73.0	4.8-89.8	0.65-0.73	0.939-1.356
(20 Rats)	3.1	96.9	129.2	10.0	35.5	40.5	0.70	1.102
<i>P Values</i>		.01-.02			.4-.5			<.01
A-B		.02-.05			.1-.2			<.01
B-C		.6-.7			.3-.4			<.01
A-C								

TABLE VI. *Riboflavin deficiency in the Rat: The level of kidney nitrogen*

Regimen		g. N/100 g. Kidney	mg. Kidney N/100g. Body wt.	Total kidney N, mg.
<b>A</b>		Ranges and Averages		
(Food controls)	...	1.9-3.0	14.2-23.8	22.4-36.8
(18 Rats)	...	2.7	21.2	31.3
<b>B</b>				
(Weight controls)	...	1.8-3.1	14.9-25.2	18.4-29.0
(20 Rats)	...	2.6	21.5	24.8
<b>C</b>				
(Riboflavin deficient)	...	1.9-2.8	16.6-25.4	18.8-30.1
(20 Rats)	...	2.5	21.4	24.4



Adams<sup>26</sup> has shown that riboflavin deficiency causes a great reduction of liver catalase, a porphyrin enzyme. Stimulation of catalase formation by testosterone also requires riboflavin<sup>26</sup>. Riboflavin causes a 5-fold increase in the synthesis of coprotoporphyrin and disappearance of porphyrin in yeast when it is added alone to the medium, whereas there was a 15-fold increase in coprotoporphyrin when both iron and riboflavin were added<sup>27</sup>. These findings indicate that riboflavin may play a role in the synthesis of porphyrin.

The activity of liver glycine oxidase, a flavin enzyme, is reduced to a considerable extent in riboflavin deprived rats<sup>15</sup> and glycine and riboflavin are both involved in the synthesis of xanthine oxidase in the liver of protein depleted rats<sup>28</sup>. Glycine has been shown to be utilised for the synthesis of protoporphyrin of hemoglobin<sup>29</sup>. This relationship between glycine and riboflavin is however far from adequate to explain the involvement of riboflavin in porphyrin synthesis from glycine.

It has been shown before, that vitamin B<sub>6</sub>, besides many other compounds of nutritional significance, is necessary for the development of cobalt polycythemia in rats<sup>30</sup> and in dogs<sup>31</sup>. The results of the experiment presented here indicate that riboflavin is also involved.

With regard to the role of riboflavin in the metabolism of protein in liver, it is of interest to note that there is a direct relationship between liver storage of riboflavin and the level of the dietary protein intake<sup>9</sup>. It has been suggested, without adequate experimental evidence, that riboflavin is mobilised in the liver where it may be primarily utilised in the metabolism of protein. There is, however, evidence that riboflavin is required for the normal utilisation of tryptophan<sup>32,33</sup>, though no enzyme mechanism has been demonstrated, as for vitamin B<sub>6</sub><sup>34</sup>.

Our experiments with the regeneration of liver nitrogen after protein starvation and hemorrhage show that riboflavin deficient rats regenerate as much liver nitrogen as do the control groups, which suggests that riboflavin insufficiency does not impair the synthesis of liver protein. Previous studies<sup>35</sup> have shown that administration of a mixture of thiamin, riboflavin, niacinamide, B<sub>12</sub> and pantothenol to rats after partial hepatectomy does not affect the rate of liver regeneration if the animals were kept on a complete diet. Rats kept on a diet deficient in the vitamin B complex showed a reduction in the total protein content, which expressed itself in an increase in the relative protein content. Our studies have shown that under conditions of riboflavin deprivation, liver maintains its normal protein level or even tends to increase it compared to the weight control group (Table V). The results of this experiment suggest a possible explanation for the role of riboflavin in protein utilisation. The inanition controls appeared to utilise, and consequently lose, liver protein, whereas the group without the vitamin did not lose any liver protein although the animals were losing weight, which might indicate that riboflavin insufficiency opposes the loss of liver protein.

It has been observed<sup>14</sup> that riboflavin is a precursor of the prosthetic group of xanthine oxidase. A rapid restoration of this enzyme activity in the liver following the administration of riboflavin to rats previously on a riboflavin deficient diet is also the result of an increase in the protein constituent.

The present study shows that the increase in the ratio of liver to body weight in riboflavin deficiency cannot be ascribed to an increase in the fat or moisture content of the liver. In other words, hypertrophy of the liver is due to a true increase of the functional



liver tissue in response to riboflavin deficiency. The significance of this finding cannot be adequately explained from the data available at present.

Studies by Roberts<sup>36</sup> on the influence of the adrenal cortex on the mobilisation of tissue protein presented evidence that the tissue protein labilised by pituitary-adrenal cortical stimulation may be utilised for accelerating liver regeneration in the partially hepatectomised rat. The enhanced repair of the liver as a result of adrenal cortical extract or adrenocorticotrophic hormone administration was characterised primarily by an increased deposition of liver protein. These results indicate that the primary effect of the adrenal cortical secretions on nitrogen metabolism involves an accelerated mobilisation of protein. The end result of the pituitary-adrenal cortical activation in this respect may be local or systemic protein anabolism or catabolism, depending upon the tissue requirements for protein at the moment. The role of cortisone as an inhibitor or regulator of protein synthesis has also been studied by Clarke<sup>37</sup>. The above observations are made because of reports on pituitary adrenal failure in the riboflavin deficient rat. Verzar and Laszt<sup>38</sup> showed that adrenalectomised rats survived less than 14 days on a riboflavin deficient diet with or without cortical hormone. If cortical hormone and yeast or riboflavin were given, or riboflavin phosphate alone, the survival time was increased to more than 2 months, whereas riboflavin alone was ineffective. Insulin sensitivity in the rat has been found to be increased in riboflavin deficiency and this has been ascribed to the impaired synthesis of adrenal glucocorticoids in riboflavin deficiency<sup>39</sup>. Guggenheim and Diamant<sup>40</sup> have shown that young rats deprived of riboflavin showed delayed diuretic response to a water load. Injection of cortisone or adrenocorticotrophic hormone increased excretion of urine by rats deprived of riboflavin, but had no effect on normal rats. Recent studies by Forker and Morgan<sup>41</sup> on the cause of pituitary-adrenal failure in the riboflavin deficient rat have led them to conclude that the defect may lie in the 'trigger' mechanism for stimulation of the pituitary to corticotrophin production rather than in the ability of the cortex to respond to corticotrophin or in the pituitary to produce corticotrophin.

This apparent relationship between the adrenal cortex and riboflavin is discussed here because it might partly answer certain questions arising from our findings, whether the effects of riboflavin deficiency on protein metabolism are primary or secondary to some other fundamental disturbance and possibly mediated through the adrenal gland.

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# PROTEIN METABOLISM IN SCURVY

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Scorbutic guinea pigs were found to excrete increased amounts of phenyl pyruvic acid, phenyl lactic acid, parahydroxyphenyl pyruvic acid, parahydroxyphenyl lactic acid and homogentisic acid<sup>1</sup>. These excretions were further enhanced when the scorbutic animals were fed tyrosine and phenylalanine<sup>1</sup>. Similar defects in tyrosine metabolism were observed in premature infants which could be corrected by the administration of ascorbic acid<sup>2,3</sup>. Scorbutic human beings and premature babies were found to excrete in urine increased amounts of amino acids<sup>4</sup>. Scorbutic monkeys showed diminished total plasma proteins<sup>5</sup>. An increase in the plasma fibrinogen content was reported in scorbutic monkeys and guinea pigs<sup>6</sup>. The above observations indicated the interrelationship between protein metabolism and vitamin C-nutrition of the body. Protein metabolism was, therefore, studied in normal and scorbutic monkeys and guinea pigs. Some of the nitrogenous constituents of blood and urine were estimated in normal and scorbutic rhesus monkeys. Different fractions of serum proteins were also estimated by paper electrophoresis in normal and scorbutic guinea pigs.

## Materials and Methods

Rhesus monkeys were fed a scorbutic diet<sup>7</sup> with 50 mg daily supplement of ascorbic acid. They were placed in individual metabolism cages and their urine collected under toluene. Total non-protein N, urea N, ammonia N, total and free amino acid N, uric acid, creatine and creatinine, total tyrosyl derivatives, ketoacids, homogentisic acid, tyrosine and phenylalanine were estimated in the 24-hour collection of urine<sup>8</sup>. Non-protein N, urea N, amino acid N and creatinine were also estimated in the blood of these animals. The animals were then fed L-tyrosine and the different tyrosyl derivatives excreted in the urine were estimated. After a few days, the animals were fed DL-phenylalanine and the urinary excretions of the above metabolites were estimated<sup>8</sup>. After the above studies were completed, the ascorbic acid supplement was withdrawn. When the monkeys developed severe scurvy, noticed 3 to 3½ months after the withdrawal of ascorbic acid supplement, the above experiments were repeated both before and after supplementation with tyrosine or phenylalanine.

Normal and scorbutic monkeys were killed by the intravenous injection of magnesium sulphate and the total tyrosyl contents of liver, kidney, stomach, muscle, small intestine and adrenals were determined. Protein content of liver was also estimated.

Scurvey was produced in guinea pigs<sup>9</sup>. Blood was withdrawn by cardiac puncture from scorbutic and paired-fed normal guinea pigs under nembutal anaesthesia. Total nitrogen and non-protein N of serum and plasma fibrinogen were determined<sup>10</sup>. Serum proteins were separated by paper electrophoresis<sup>11</sup>. The results are given in Tables I-IV.



TABLE I  
*Urinary excretion of non-protein nitrogenous constituents by normal  
 and scorbutic Rhesus monkeys*  
 (Average of four monkeys)

Urinary constituents	Normal (mg/kg/24 hr)	Scorbutic (mg/kg/24 hr)
Ammonia N ... ..	53 ± 9	78 ± 10
Urea N ... ..	245 ± 50	563 ± 102
Total non-protein N ... ..	348 ± 54	761 ± 104
Free amino acid N ... ..	3.2 ± 0.4	8.4 ± 1.8
Total amino acid N ... ..	32 ± 4	69 ± 2
Uric acid ... ..	2.0 ± 0.3	3.9 ± 0.4
Creatinine ... ..	33 ± 4	32 ± 3
Creatine ... ..	absent	22 ± 1

TABLE II  
*Some of the nitrogenous constituents of tissues of normal and scorbutic monkeys*  
 (Average of four monkeys)

Constituents	Tissues	Unit of values	Normal	Scorbutic
Total tyrosyl derivatives of	kidney	mg/g. fresh	0.70 ± 0.03	1.20 ± 0.12
	liver	„ tissue	0.47 ± 0.03	0.78 ± 0.02
	stomach	„	0.36 ± 0.18	1.30 ± 0.12
	small intestine	„	0.76 ± 0.09	1.17 ± 0.12
	adrenals	„	1.21 ± 0.05	1.28 ± 0.09
Protein of ... ..	liver	„	274 ± 8	199 ± 2
Non-protein N of ... ..	whole blood	mg/100 ml	34 ± 3	113 ± 2
Urea N of ... ..	„	„	22 ± 2	100 ± 4
Amino acid N of ... ..	„	„	9 ± 1	...
Creatinine of ... ..	„	„	1.5 ± 0.1	2.3 ± 0.2

## Results and Discussion

There was a marked increase in the non-protein N of blood and in the urinary excretion in scorbutic monkeys. Scorbutic guinea pigs also showed increased NPN of blood serum. These observations might be due to decreased utilization, increased catabolism or both of protein in scurvy. Blood urea N in scurvy formed 90 per cent of the total NPN. The same value in normal monkeys was 64 per cent. The increased elimination of urea N in urine is suggestive of increased protein catabolism which, however, is contradictory to the view<sup>12</sup> that ascorbic acid stimulates arginase in the liver leading to increased production of urea. No significant change in the uric acid excretion in scurvy might indicate that ascorbic acid is not concerned with purine metabolism in intact animal. Blood creatinine increased in scorbutic monkeys, but creatinine excreted in the urine did not change. Creatine, which was absent in the urine of normal animals, appeared in the urine of scorbutic monkeys. The main cause for the elimination of creatine in the urine of scorbutic monkeys might be due to associated muscular dystrophy which is likely to lead to the inability of muscle to withdraw creatine from blood. Another possibility might be that less high



TABLE III  
*Urinary excretions of total tyrosyl derivatives, homogentisic acid, keto acids, tyrosine and phenylalanine by Rhesus monkeys*  
(Average excretion\* by five monkeys)

Treatment given	Metabolites excreted (as % of ingested tyrosine or phenylalanine)	Normal	Scorbutic
Animals ingested tyrosine and phenylalanine present in the scorbutic diet	Total tyrosyls	3.1 ± 0.3	12.9 ± 3.6
	Keto acids	1.1 ± 0.2	2.0 ± 0.5
	Homogentisic acid	0.21 ± 0.02	0.9 ± 0.46
	Tyrosine	0.11 ± 0.06	0.42 ± 0.09
2 g. L-tyrosine fed for 2 days. Total supplement = 4 g.	Total tyrosyls	7.1 ± 0.3	50.0 ± 6.1
	Keto acids	2.7 ± 0.3	26.2 ± 2.2
	Homogentisic acid	0.58 ± 0.07	12.6 ± 1.8
	Tyrosine	1.18 ± 0.10	3.7 ± 0.9
	Phenylalanine	0.64 ± 0.09	...
1 g. DL-phenylalanine fed for 2 days. Total supplement = 2 g.	Total tyrosyls	4.0 ± 0.5	12.1 ± 3.9
	Keto acids	2.7 ± 0.1	19.0 ± 5.0
	Homogentisic acid	0.47 ± 0.05	4.4 ± 2.8
	Phenylalanine	1.7 ± 0.1	2.2 ± 0.4
	Tyrosine	0.18 ± 0.10	0.64 ± 0.17

\* Results are expressed as means ± standard error of the mean.

TABLE IV  
*Different fractions of serum proteins of guinea pigs (%)*

Serum protein fractions					Normal (10)	Scorbutic (8)	t
Albumin	...	...	...	...	2.82 ± 0.05	1.90 ± 0.08	9.86
α <sub>1</sub> -globulin	...	...	...	...	0.42 ± 0.03	0.77 ± 0.03	8.88
α <sub>2</sub> -globulin	...	...	...	...	1.38 ± 0.08	1.19 ± 0.06	2.00
β <sub>1</sub> -globulin	...	...	...	...	0.35 ± 0.01	0.31 ± 0.03	1.25
β <sub>2</sub> -globulin	...	...	...	...	0.56 ± 0.03	0.43 ± 0.02	3.93
γ-globulin	...	...	...	...	0.68 ± 0.05	0.69 ± 0.04	0.19
Total serum protein	...	...	...	...	6.2 ± 0.10	5.29 ± 0.17	4.6
Non-protein nitrogen	...	...	...	...	0.06 ± 0	0.10 ± 0.01	3.3
Plasma fibrinogen	...	...	...	...	0.31 ± 0.04	0.56 ± 0.07	3.2

Figures in parenthesis indicate the number of animals used.

energy phosphates were available in scurvy for phosphorylation of free creatine. It has been shown<sup>13-16</sup> that ascorbic acid deficiency hampers the reactions through the Krebs tricarboxylic acid cycle. Hence, the main energy generating system of the body might be deranged in scurvy, decreasing thereby high energy phosphate supply to form creatine phosphate and to retain it in tissues. The protein content of liver decreased in scorbutic monkeys indicating a fundamental role of ascorbic acid in synthesizing liver proteins.

The normal monkeys excreted in urine very little tyrosyl derivatives, but when the animals developed scurvy these excretions increased considerably. With a load of tyrosine



and phenylalanine, the excretions of tyrosyls increased in normal monkeys but in scorbutic monkeys, under similar treatment, the excretions were further enhanced. The increased urinary excretion of homogentisic acid as observed in scorbutic monkeys and in scorbutic guinea pigs<sup>1</sup> and the absence of this substance in the urine of premature infants<sup>3</sup>, possibly indicates that the metabolism of tyrosine differs in different species of animals. Urinary excretion of tyrosine also increased in scorbutic monkeys, but the increase was not as marked as the excretions of its metabolites. Ascorbic acid seems to have no specific action on the conversion of tyrosine to parahydroxyphenyl pyruvic acid in monkeys. The increase in the keto acid in scurvy might lead to the reverse reaction by mass action with a consequent increase in tyrosine. The site of action of ascorbic acid in tyrosine metabolism in rhesus monkeys appears to be after the formation of keto acid and homogentisic acid, indicating that ascorbic acid is needed in attacking the side chain as well as the ring of tyrosine molecule. Increased urinary excretion of tyrosine metabolites, after phenylalanine was fed, indicated that phenylalanine is converted to tyrosine. Relatively higher excretion of keto acids compared to total tyrosyl derivatives was obtained in scorbutic monkeys after phenylalanine was fed which possibly indicated that ascorbic acid is concerned in the oxidation of phenylpyruvic acid and in the oxidative degradation of tyrosine. No significant increase in the urinary excretion of tyrosine after the feeding of phenylalanine was observed in scorbutic monkeys. Ascorbic acid, therefore, is not responsible for the continued conversion of phenylalanine to tyrosine. The accumulation of tyrosyl derivatives in the liver, kidney, muscle and small intestine of scorbutic monkeys seems to be due to the lowered capacity of these tissues to oxidize tyrosine.

Six clear components of serum proteins were present in guinea pig serum. Serum albumin,  $\alpha_2$ - and  $\beta_2$ -globulin values decreased considerably and significantly,  $\alpha_1$ -globulins increased significantly and there was no change in  $\gamma$ -globulin and  $\beta_1$ -globulin fractions when the guinea pigs developed scurvy. Antibodies are mainly contained in the  $\gamma$ -globulins of plasma proteins<sup>17</sup>.  $\gamma$ -globulin values, however, did not change in scorbutic guinea pigs although it has been stated<sup>18</sup> that vitamin C-nutrition influences the development of immunity in the body. Elevation of fibrinogen in scurvy might be due to dysfunction of the liver. The changes in the composition of plasma proteins in scurvy indicate the interrelations of vitamin C-nutrition and protein metabolism in the body.

## Summary

In scorbutic rhesus monkeys, blood levels of non-protein N, urea N and creatinine were found to be increased. These animals also excreted increased amounts of non-protein N, urea N and amino acid N in urine. Creatine, which was absent in urine of normal monkeys, appeared in the urine of scorbutic animals with increased amounts of total tyrosyl derivatives, keto acids, homogentisic acid and tyrosine, and these excretions were further enhanced when they were fed tyrosine or phenylalanine. Protein content of livers of scorbutic monkeys was less than normal. Liver, kidney, muscle and small intestine of scorbutic monkeys contained more tyrosyls, suggesting inefficient oxidation of tyrosine in ascorbic acid deficiency. Paper electrophoretic studies with serum of scorbutic and paired-fed normal guinea pigs showed a significant decrease in albumin,  $\alpha_2$ - and  $\beta_2$ -globulins, considerable increase in  $\alpha_1$ -globulin and no significant change in  $\beta_1$ - and  $\gamma$ -globulins in the serum of scorbutic guinea pigs. Total serum proteins of scorbutic guinea



pigs were low and serum non-protein N and plasma fibrinogen were high. The above changes observed in scurvy indicate the interrelations of vitamin C-nutrition and protein metabolism in this animal.

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# INFLUENCE OF DIETARY PROTEIN LEVELS ON THE VITAMIN C STATUS OF THE RAT

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In the course of some studies on protein deficiency, we made the casual observation that rats on high protein diets excreted much larger amounts of total ascorbic acid in the urine than those on low protein diets. A number of reports in the literature suggest a possible relationship between dietary protein and the vitamin C status of experimental animals. Thus, Cohen and Mendel<sup>1</sup> observed that susceptibility to scurvy in the guinea pig was more on diets low in protein. Ray<sup>2</sup> reported that the real precursor of ascorbic acid was a substance different from the hexoses. Later, Hopkins and Slater<sup>3</sup> suggested that proteins could be the possible precursors. Wallace and Zilva<sup>4</sup> had also assumed a nitrogenous precursor. Sherman and Smith observed that aged guinea pigs succumbed more rapidly to scurvy and especially when the dietary protein was of poor quality. Straumfjord and West<sup>6</sup> found that in the alloxan diabetic rat ascorbic acid excretion was more when they were on a diet of evaporated milk.

Also, Chakravorthy and Roy<sup>7</sup> recorded a significantly higher excretion of ascorbic acid in human beings receiving a fat or a protein diet as compared with the normals. Ahmed<sup>8</sup> recorded higher urinary excretion of vitamin C in humans fed animal proteins. Later, Heinemann<sup>9</sup> showed that in Ahmed's experiment the possibility of thiosulphate simultaneously excreted in the urine on high protein diets being responsible for the high dye titre had not been excluded.

In view of the above observations it was considered desirable to re-examine the influence of dietary protein level on vitamin C excretion in the rat, in the first instance, employing the more recently developed dinitrophenylhydrazine method of Roe and Keuther<sup>10</sup> for the estimation of urinary ascorbic acid, as this method is now generally accepted as a more specific measure of ascorbic acid than the rather ambiguous methods based on dye reduction.

## Materials and Methods

Adult male albino rats with body-weights in the range 160 g to 180 g were employed in all the experiments. They were fed synthetic diets containing varying amounts of casein (Polson's), 10 per cent fat, optimal amounts of vitamins (other than vitamin C) and minerals.

Urinary excretion and tissue levels of ascorbic acid (total) were determined by the Roe and Keuther method<sup>10</sup>. The identity of the dinitrophenyl hydrazone derivative in the urine with that of standard ascorbic acid was established by similarity in chromatographic behaviour, and absorption spectra of the hydrazones prepared from the urine of rats (especially those with very high excretion) and from standard ascorbic acid (Merck).

Urine was collected in 10 per cent trichloroacetic acid over a period of 3 days, collecting funnels and the bottoms of the cages (of wide mesh, coated with resistant paint) being



washed at least thrice during each day of the collection period. In most cases the urine was collected over a three-day period, made up to known volumes and aliquots used for the vitamin C determination. Under the experimental conditions almost all the ascorbic acid in the urine was in the oxidized form (DHA & DKA). In a few test cases, urine was collected in 5 per cent metaphosphoric acid solution overnight and ascorbic acid was estimated both by dye reduction and Roe and Keuther's method. The results indicated that at least 60 per cent of the ascorbic acid excreted by the rat was in the reduced form.

## Results

In the first experiment, stock rats (distributed into groups of 6 each according to randomised block design) were transferred to diets containing 10, 30 and 50 per cent casein. Urinary ascorbic acid excretions were measured after the rats had been on the synthetic diets for two weeks. The results are given in Table I.

TABLE I

*Urinary ascorbic acid excretion of rats transferred from stock diet to adequate synthetic casein diets*

Period of feeding: 3 weeks

No. of animals in each group: 6

Group		Dietary protein level	Urinary ascorbic acid (av. mg/24 hrs)
I	...	10%	1.18
II	...	30%	2.10
III	...	50%	2.87

$\pm 0.19$   
(15 d.f.)

Statistical significance I ~ II\*; I ~ III†  
II ~ III‡

\* Significant at 5% level; † Significant at 1% level; ‡ Significant at 0.1% level; N.S.: not significant.

In the next experiment, randomised groups of adult male rats (groups of 5 each) were fed a protein-free diet for two weeks and later transferred to synthetic diets containing various levels of casein and the urinary excretion of ascorbic acid in these groups of rats was measured after a two/three week refeeding period. The results are given in Table II.

The results showed that rats receiving the 10 per cent casein diet excreted nearly twice as much vitamin C as those fed the protein-free diet and those on the 30-50 per cent protein diets excreted twice as much as those on the 10 per cent protein diet. The urinary excretion by animals on 30-50 per cent casein diets was not significantly different. The tissue levels—liver, blood and adrenals—were not very much influenced by the protein content of the diets.

In view of the results reported by Schwartz, *et al.*<sup>11</sup> that aminopterine and sulphasuxidine ingested with the diets markedly reduced liver levels and urinary excretion of ascorbic acid in the rat, the effect of the inclusion of 2 per cent sulphaguanidine in the low protein



TABLE II

Urinary ascorbic acid excretion of rats fed adequate diets containing various levels of casein after a 2 week protein depletion

Period of feeding: 3 weeks                      No. of animals in each group: 5

Group		Dietary protein level (per cent)	Urinary ascorbic acid av. for 24 hrs. (mg)
I	...	0	0.48
II	...	10	1.28
III	...	30	2.44
IV	...	50	2.92

$\pm 0.30$   
(8 d.f.)

Statistical significance: I ~ II\*; II ~ IV†;  
II ~ III†; III ~ IV N.S.

(10 per cent) as well as the high protein (50 per cent) diets on the urinary ascorbic acid excretion of groups of adult male rats (10 in each group) was examined. The results (Table III) on the low protein diet showed that the sulpha drug considerably reduced the urinary excretion as well as the liver levels of the vitamin. This adverse effect of the sulpha drug was very much reduced when the protein content of the diet was high.

TABLE III

Effect of sulfaguanidine in the diet on urinary excretion and tissue levels of ascorbic acid in the rat  
No. of animals in each group: 10

Diet				Period of feeding (weeks)	Total Ascorbic Acid				
					Urinary excretion Av. mg/24 hrs	Liver		Adrenals Total $\mu$ g (Av.)	Blood mg/100ml (Av.)
						Total mg	mg/g fresh wt		
10% casein diet	...	...	...	6	1.6	3.4	0.38	85.7	1.24
10% casein diet; sulfaguanidine 2%	...	...	...	6	0.35	1.4	0.25	84.5	0.76
50% casein diet	...	...	...	5	1.5	...	...	...	...
do	...	...	...	10	1.5	2.74	0.38	105.9	1.24
50% casein diet; sulfaguanidine 2%	...	...	...	5	1.5	...	...	...	...
do	...	...	...	10	1.3	2.48	0.38	107.6	1.38

Statistical significance: Differences in urinary excretion, liver and adrenal levels of the vitamin between groups of rats fed the low protein diet with and without the sulfa-drug are significant at 1% level whereas these differences in animals receiving the high protein diet are not significant. As blood specimens were either lost in some cases or could not be obtained in some others, the differences have not been statistically evaluated and only averages are given.



## Discussion

The evidence presented suggests the possibility of dietary protein levels influencing the *in vivo* synthesis of ascorbic acid in the rat either by increasing the liver content of the enzymes concerned in the biosynthesis of the vitamin or by providing a high concentration of precursors. The latter may involve directly or indirectly, the intestinal microflora. If these effects with high protein diets and the anti-bacterial agents are also obtained with primates, they would have considerable significance to the vitamin C nutrition of man. Experiments along these lines are in progress.

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## Discussion

- Q. What proof is there that the Roe and Keuther-chromogen in the urine represents true total ascorbic acid?
- A. The DNPH-derivatives prepared from urines showing high ascorbic acid content were identical with the derivative from standard ascorbic acid in respect of behaviour on paper chromatograms developed with a number of solvents, melting point and absorption spectrum. There was no evidence of the presence of any DNPH-derivative other than that which corresponded to the derivative from ascorbic acid. So, the chromogen as measured must represent true total ascorbic acid and closely related metabolic products.
- Q. Could the effect of the sulfa-drugs be ascribed to any influence on the ascorbic acid synthesising enzymes of the liver?
- A. Our preliminary observations are to the contrary and appear to implicate the intestinal microflora directly or indirectly in the biosynthesis of ascorbic acid in the rat, presumably through providing precursors.
- Q. Then, what would be the interpretation of the results with high protein diets?
- A. High dietary protein levels may enhance both the levels of liver enzymes and provision of precursors.



# A STUDY OF THE EFFECTS OF VARIATIONS IN PROTEIN QUALITY AND PROTEIN LEVEL ON GROWTH AND BODY COMPOSITION OF RATS\*

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The low nutritive value of vegetable proteins as compared to animal proteins is attributable to imperfection in the amino acid make-up of the vegetable protein and may be corrected by proper fortification of the protein with the limiting amino acids. Increasing the level of low quality vegetable protein in the diet may lead to a level at which the growth response obtained is equal to that obtained with the animal protein diet at a lower level. It has been reported that peanut protein is equal to casein in promoting growth of rats when fed at 20 per cent level<sup>1,2</sup>. Consequent on the higher intake of low quality protein, certain other changes may occur. It has been observed that with higher intakes of protein, there is a decrease in protein efficiency ratio which has been attributed to a greater waste in metabolism<sup>3</sup>. Stanier<sup>4</sup> noted that when rats were fed a high protein diet in such a way that their body weight ran parallel to that of animals on a low protein diet, certain changes in body composition were caused.

A preliminary experiment was, therefore, designed to ascertain the level at which a low quality protein, such as wheat gluten, would give the same growth response as given by a high quality protein, such as casein and to study the effect of these proteins on body composition.

## Experimental

The animals were fed the respective diets (Table I) for a period of 12 weeks during which a careful record of the growth and food intake was maintained. At the end of the experimental period, the animals were sacrificed under ether anaesthesia and blood withdrawn from the portal vein and immediately heparinized. Liver, kidney, spleen, heart and lung were removed for analysis. For determining the carcass composition the method of Bender and Doell<sup>5</sup> was adopted. The carcass was carefully cut open, weighed and then dried to constant weight in an air oven at 105°. The dried carcasses were powdered and extracted with ether in a Soxhlet apparatus for 24 hours. After evaporation of the solvent, the extracted fat was dried at 105°. Nitrogen was determined in the defatted carcass by the Kjeldahl method.

A portion of the heparinized blood was immediately used for cell count and haemoglobin determinations, while the rest was centrifuged in the cold to obtain the plasma. Haemoglobin was determined by the acid hematin method. The organs after removal were cleaned of connective tissues, weighed and 20 per cent homogenates in ice-cold distilled water were prepared using a Potter-Elvehjem glass homogeniser. Aliquots of the homogenates were used for various determinations.

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TABLE I  
Effect of variations in protein quality and protein level on growth and food intake

Group	Diet	Average gain weight (g) during				Average daily food intake (g)
		2 weeks	4 weeks	8 weeks	12 weeks	
1	Casein (10 per cent protein)	26.6	45.0	75.6	85.6	7.6
		$\pm 2.7$	$\pm 3.2$	$\pm 3.3$	$\pm 4.7$	$\pm 0.3$
2	Wheat gluten (12 per cent protein)	11.0	17.3	35.5	52.3	6.1
		$\pm 1.8$	$\pm 2.4$	$\pm 2.9$	$\pm 3.8$	$\pm 0.4$
3	Wheat gluten (15 per cent protein)	15.3	24.6	45.0	64.3	6.6
		$\pm 2.1$	$\pm 3.1$	$\pm 2.5$	$\pm 3.7$	$\pm 0.2$
4	Wheat gluten (18 per cent protein)	20.3	38.0	68.5	80.0	7.2
		$\pm 2.7$	$\pm 3.8$	$\pm 3.4$	$\pm 4.2$	$\pm 0.2$

Male albino rats (Wistar strain) were divided into four groups and fed *ad libitum* the following diets for 12 weeks: (1) casein at 10 per cent protein level and (2), (3) and (4) wheat gluten at 12 per cent, 15 per cent and 18 per cent protein levels. Additions common to all diets were (percentages): sesame oil (containing synthetic vitamin A palmitate 100 I.U./g.; and  $\alpha$ -tocopherol 1 mg/g) 5; salt mixture, U.S.P. XIV, 4; Vitaminised sucrose 1; and maize starch in appropriate amounts. Vitaminised sucrose contained per g; thiamine hydrochloride, 0.30 mg; riboflavin 0.40 mg; pyridoxine hydrochloride, 0.30 mg, Ca-d-pantothenate, 2.0 mg; nicotinic acid, 2.0 mg; inositol, 50.0 mg; p-amino benzoic acid, 10.0 mg. biotin, 0.1 mg; Vit. B<sub>12</sub> 0.015 mg; and folic acid 0.10 mg. choline chloride and Vit. K (menadione) were added to the diets separately at levels of 500 mg/kg. and 20 mg/kg. respectively.

Results are averages of 4 independent samples  $\pm$  S.E.M.

Protein-free plasma filtrates were prepared according to the method of Hier and Bergeim<sup>6</sup>; those of liver and other organs were obtained using 10 per cent trichloroacetic acid for precipitation of proteins. Nitrogen in plasma and tissue homogenates and in their protein-free preparations was determined by the micro-Kjeldahl method<sup>7</sup>.

Total lipids were determined according to the ethanol-ether extraction procedure of Stekol<sup>8</sup>. For determination of phospholipids, the total lipids obtained as above were digested with 30 per cent sulphuric acid until a colourless solution was obtained. A drop of concentrated nitric acid was then added and the solution heated for about 5 minutes. This was allowed to cool and diluted with distilled water. Aliquots of this solution were taken for estimation of phosphorus by the method of Taussky and Shorr<sup>9</sup>. The phospholipids were calculated by using 25 as a factor for conversion of phosphorus to phospholipids.

Cholesterol in liver and plasma was determined by the method of Schoenheimer and Sperry<sup>10</sup>.

For separation of  $\alpha$ - and  $\beta$ -lipoprotein complexes in plasma by paper electrophoresis and subsequent quantitation of total lipid, total cholesterol and phospholipid in these complexes, the method of Brown *et al.*<sup>11</sup> was followed. Separation was achieved by electrophoresis of plasma on Whatman No. 3 MM paper strips using barbital buffer solution (pH 8.6 and ionic strength 0.75) at a constant current of 2.5 mA per strip in a horizontal open strip type cell for 18 hours at room temperature (28°). The strips were then dried, stained in a solution of bromophenol blue, washed with 2 per cent acetic acid followed by distilled water and cut into two portions between the  $\alpha$ - and  $\beta$ -lipoprotein components.



The two portions of each strip were dried at room temperature. Each portion was then cut into small pieces and placed in 10 ml boiling water. After evaporating off nearly to dryness, the band was eluted with hot solution of alcohol and acetone (1:1). The eluates derived from the  $\alpha$ - and  $\beta$ -lipoprotein complexes respectively were divided into three aliquots for determination of total cholesterol, phospholipids and total lipids by the methods mentioned above.

## Results and Discussion

Results for growth and food intake are given in Table I and data on carcass composition are summarised in Table II. It is seen that on the wheat gluten diets at 12 and 15 per cent protein levels, growth is rather poor and only when the protein level is increased to 18 per cent, a growth response almost equal to that with the 10 per cent casein diet is obtained. Food intake on the wheat gluten diets is also low and increases with the increase in protein level of the diet.

TABLE II  
*Effect on carcass composition*

Group	g./100 g. fresh weight		
	Water	Fat	Nitrogen
1	67.5 ± 0.7	8.4 ± 0.3	3.21 ± 0.08
2	68.2 ± 0.9	12.1 ± 0.6	2.32 ± 0.03
3	65.7 ± 0.5	13.5 ± 0.4	2.58 ± 0.05
4	62.2 ± 0.8	15.3 ± 0.7	2.91 ± 0.07

The weight gain in animals on the low quality protein diet is probably mainly fat rather than protein (Table III). It can be seen that the carcass lipid content on the 18 per cent protein diet is almost double that on the 10 per cent protein diet, while the nitrogen content is somewhat lower. Stanier<sup>4</sup> studying the carcass composition of rats fed a high protein diet in such a way that their mean body weight ran parallel to that of animals on a low protein diet, observed that the carcasses of the animals on the high protein diet had much less fat than those on the low protein diet.

Results for analyses of liver and other organs are given in Table III. The changes in body weight are reflected in the results obtained for nitrogen, lipids and phospholipids in the various organs. The protein content of the liver is, to a certain extent, a function of the quantity and nutritive value of dietary protein<sup>12</sup>. This is borne out from the observation that liver nitrogen values are lowered on the 12 and 15 per cent protein diets, while on the 18 per cent diet values almost equal to that on the 10 per cent casein diet are obtained. Liver non-protein nitrogen values are rather high on diets 2 and 3 but are reduced on diet 4.



TABLE III  
*Changes in nitrogen, lipids and phospholipids in tissues*

Organs	mg/g. fresh weight															
	Total nitrogen				Non-protein nitrogen				Total lipids				Phospholipids			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
...	34.9 ±1.1	24.7 ±4.5	28.2 ±4.1	33.0 ±2.3	2.4 ±0.09	3.05 ±0.20	2.89 ±0.12	1.87 ±0.17	64.9 ± 3.0	81.1 ± 8.2	70.5 ±11.0	58.0 ± 7.0	29.1 ± 2.1	21.2 ± 1.2	23.3 ± 1.7	26.8 ± 2.5
...	29.6 ±3.4	20.8 ±0.2	22.6 ±1.8	26.0 ±2.0	2.18 ±0.02	3.66 ±0.16	2.92 ±0.59	2.34 ±0.10	62.6 ± 5.1	61.6 ± 7.0	59.0 ± 4.1	50.2 ± 3.0	20.3 ± 0.6	19.2 ± 0.3	22.2 ± 1.3	18.8 ± 1.8
...	22.6 ±1.1	17.4 ±1.6	18.7 ±1.2	20.7 ±3.5	1.79 ±0.39	3.32 ±1.0	1.68 ±0.18	1.45 ±0.22	59.4 ± 5.8	64.5 ± 3.0	61.7 ± 5.5	65.5 ± 2.7	18.8 ± 0.6	16.3 ± 1.0	17.8 ± 0.3	19.3 ± 0.8
...	30.5 ±2.5	24.7 ±0.8	24.8 ±1.2	29.5 ±1.5	2.92 ±0.21	3.12 ±0.40	2.28 ±0.54	2.55 ±0.19	58.5 ± 4.2	60.4 ± 3.1	51.2 ± 5.1	56.4 ± 8.0	18.2 ± 0.9	17.9 ± 1.9	17.4 ± 0.8	18.1 ± 0.6
...	29.7 ±0.3	28.0 ±1.2	23.5 ±1.1	32.1 ±1.1	2.59 ±0.33	2.65 ±0.42	2.63 ±0.53	2.73 ±1.0	63.4 ± 3.2	54.8 ± 0.8	60.3 ± 2.3	58.6 ± 4.4	21.4 ± 0.7	17.5 ± 2.0	23.9 ± 0.8	25.3 ± 1.6

For details see footnote, Table I.

Increased fat in the liver is seen in group 2 which is lowered with increasing protein content of the diet. The reverse trend is observed in the case of phospholipids. On the whole, it may be noted that the results obtained for liver and other organs on the wheat gluten diet at 18 per cent protein level approximate most closely to the results obtained on the 10 per cent casein diet. A study of the organ: body ratios (Table IV) also leads to the same

TABLE IV  
*Organ: body ratios for liver, heart, lungs, spleen and kidneys*

Group	$\frac{\text{Organ weight}}{\text{Body weight}} \times 100$				
	Liver	Heart	Lungs	Spleen	Kidneys
1	3.14 ±0.12	0.361 ± 0.003	0.528 ± 0.038	0.290 ± 0.006	0.635 ± 0.04
2	4.41 ±0.51	0.413 ± 0.017	0.659 ± 0.04	0.375 ± 0.02	0.830 ± 0.05
3	3.86 ±0.72	0.379 ± 0.015	0.661 ± 0.006	0.302 ± 0.02	0.768 ± 0.01
4	3.31 ±0.26	0.370 ± 0.027	0.581 ± 0.013	0.289 ± 0.02	0.718 ± 0.03

For details see footnote, Table I.



conclusion. While the organ: body ratios are distinctly higher on the 12 and 15 per cent protein diets, those on the 18 per cent diet are nearly equal to those on the 10 per cent casein diet.

Table V gives the results of the blood analyses. As may be expected, there is a reduction in haemoglobin and other constituents on the low quality protein diets as compared to casein. Mulgaonkar<sup>13</sup> observed that animals on a casein ration show relatively higher haemoglobin concentration compared to those fed a peanut protein diet. Results obtained for plasma nitrogen and lipids (Table VI) are more or less similar to the results obtained for liver and other organs.

TABLE V. *Blood picture*

Group		Haemoglobin g/100 ml	Leucocytes/mm <sup>3</sup> ( $\times 10,000$ )	Erythrocytes/mm <sup>3</sup> ( $\times$ million)
1	...	14.7 $\pm 0.8$	0.75 $\pm 0.18$	6.3 $\pm 0.6$
2	...	13.3 $\pm 0.5$	0.62 $\pm 0.09$	5.2 $\pm 0.3$
3	...	13.6 $\pm 0.3$	0.66 $\pm 0.13$	5.5 $\pm 0.4$
4	...	13.8 $\pm 0.6$	0.68 $\pm 0.11$	5.8 $\pm 0.7$

For details see footnote, Table I.

TABLE VI. *Changes in plasma nitrogen, lipids and phospholipids*

Group		mg./100 ml. of plasma			
		Total nitrogen	Non-protein nitrogen	Total lipids	Phospholipids
1	...	922 $\pm 61$	43.6 $\pm 2.9$	264 $\pm 12$	187.5 $\pm 12.0$
2	...	691 $\pm 9$	62.6 $\pm 6.0$	340 $\pm 24$	106.2 $\pm 5.2$
3	...	700 $\pm 10$	62.0 $\pm 4.2$	292 $\pm 12$	131.2 $\pm 19$
4	...	872 $\pm 29$	49.6 $\pm 6.3$	287 $\pm 15$	170.8 $\pm 29$

For details see footnote, Table I.

Results for cholesterol in liver and plasma are given in Table VII. Table VIII gives the data on lipid distribution in  $\alpha$ - and  $\beta$ -lipoprotein fractions of plasma. No significant differences in cholesterol levels are observed on feeding the different diets. Although cholesterol levels are known to be affected by protein level and protein quality<sup>14, 15, 16</sup> the absence of any significant changes in this experiment may be due to the short dura-



TABLE VII  
*Changes in liver and plasma cholesterol*

Group		Liver (mg/100 g. fresh wt)		Plasma (mg/100 ml)	
		Free	Total	Free	Total
1	...	195 ± 7	306 ± 12	14 ± 1.3	62 ± 3.2
2	...	202 ± 11	312 ± 9	12 ± 0.7	65 ± 1.8
3	...	168 ± 8	302 ± 13	15 ± 0.4	61 ± 4.1
4	...	192 ± 5	296 ± 15	16 ± 1.1	56 ± 2.9

For details see footnote, Table I.

TABLE VIII  
*Lipid distribution in  $\alpha$ - and  $\beta$ -lipoproteins of plasma*

Group		Total cholesterol (mg/100 ml)			Total lipids (mg/100 ml)			Phospholipids (mg/100 ml)		
		$\alpha$ -	$\beta$ -	%in $\beta$ -portion	$\alpha$ -	$\beta$ -	%in $\beta$ -portion	$\alpha$ -	$\beta$ -	%in $\beta$ -portion
1	...	20 ± 1.2	38 ± 1.7	65.5	115 ± 3.2	180 ± 4.2	61.1	96.3 ± 2.6	77.9 ± 3.7	44.7
2	...	20 ± 0.7	41 ± 2.2	67.2	125 ± 2.8	160 ± 2.8	56.2	61.1 ± 1.3	57.5 ± 1.9	48.4
3	...	22 ± 0.6	37 ± 0.6	62.7	130 ± 4.1	150 ± 1.8	53.6	61.8 ± 1.8	61.0 ± 2.4	49.6
4	...	21 ± 1.4	34 ± 1.3	61.8	115 ± 2.6	160 ± 3.1	58.2	89.7 ± 3.4	78.4 ± 3.6	46.6

For details see footnote, Table I.

tion of the experiment and to the fact that the fat used was sesame oil at a level of 5 per cent only, instead of saturated fat at a higher level. These results are also reflected in the concentrations of lipids, phospholipids and cholesterol in the  $\alpha$ - and  $\beta$ -lipoproteins of plasma. The values obtained are all within the normal range and no changes are seen with the different diets.

From the data obtained in this experiment, it would appear that gluten at a level of 18 per cent produces almost the same results as casein at 10 per cent. However, the weight gain on the 18 per cent protein diet is mostly body fat probably due to imperfections



in the amino acid make up of the wheat gluten. Further experiments are proposed to be carried out to study the effect of supplementing these two diets with B vitamins at different levels.

### Summary

Rats were fed a 10 per cent casein diet and wheat gluten diets with 12, 15 and 18 protein to determine the level at which wheat gluten gave the same growth response as casein and to study the effects of these proteins on body composition.

It was observed that wheat gluten at 18 per cent level in the diet gave a growth response almost similar to that given by 10 per cent casein. A study of the carcass composition revealed that the gain in weight was chiefly body fat.

Analysis of liver and other organs for nitrogen, lipids and phospholipids indicated that on the 18 per cent wheat gluten diet, values were obtained which closely approximated to those on the 10 per cent casein diet.

Blood analysis showed a decrease in haemoglobin and other constituents on the gluten diets. A study of liver and plasma cholesterol and total lipids, phospholipids and total cholesterol in the  $\alpha$ - and  $\beta$ -lipoproteins of plasma did not reveal significant differences.

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# EFFECTS OF PROTEIN PER CALORIE RATIO, DIETARY FAT LEVEL AND B VITAMINS ON PROTEINS AND CALORIE UTILIZATION IN THE GROWING RAT

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Reports pertaining to the influence of the source of non-protein calories on nitrogen metabolism have been contradictory. Maignon<sup>1</sup> in a review of the literature pointed out that many early workers, notably Voit and Atwater, reported improved protein utilization when protein-carbohydrate mixtures were fed as opposed to protein fat combinations. His own experimental work, however, indicated that in diets of moderate egg albumin content, carbohydrate and fat exerted similar effects but that fat was superior when protein was increased. This led him to suggest that fat has a specific role in protein metabolism apart from the energy supplied. Deuel<sup>2</sup> has reviewed the non-calorific functions of fat in the diet. On the other hand, an extensive review of the efficacies of fat and carbohydrate as protein spacers under a variety of experimental conditions suggested to Munro<sup>3</sup> that carbohydrate has a specific role in the utilization of dietary protein, for which energy in the form of fat is not a substitute. According to Yoshida and co-workers<sup>4</sup> the results of experimental work in this field could be grouped under four different categories. In the first place, nitrogen retention is influenced by the caloric intake of the animal. Rosenthal and Allison<sup>5</sup> suggest that there may be an optimum caloric intake for each level of dietary protein. Experiments on moderately undernourished animals, on animals receiving adequate diets and on surfeit-fed animals suggest that, as a source of calories, fat and carbohydrate are equally effective in sparing protein in mature animals when the diet contains all the three components<sup>6-8, 3, 9-10</sup>. Secondly, evidence from experiments in which animals have been fed carbohydrate-free or protein-free diets suggests that carbohydrate plays a role in protein metabolism which cannot be taken by fat<sup>6, 8, 11, 12</sup>. Thirdly, Swanson<sup>8</sup> and Deuel<sup>7</sup> have presented evidence for a specific nitrogen sparing effect of fat under conditions of severe caloric restriction. In contrast, Calloway and Spector, in shorter experiments on dogs, observed no specific beneficial effect of fat. Finally, there is no evidence, according to Munro<sup>3</sup>, to indicate that there are differences in protein utilization during growth due to the carbohydrate or fat content of diet. This view is generally supported by the observations of Forbes and associates<sup>13</sup>, French, Block and Swift<sup>14</sup> and Metta and Mitchell<sup>10</sup>.

More recent interest in this field has centered around studies on protein utilization in relation to the proportion of protein to calories in the diet. The protein per calorie ratio has been recognised as an important factor in poultry nutrition<sup>15-20</sup>. Donaldson, Combs and Rosomoser<sup>21</sup> cited evidence from the literature, and from their own experiments on chicks, to show that growth, calorie utilization, protein utilization and carcass composition were influenced by the protein per calorie ratio of the diet. Schreiber and Elvehjem<sup>22</sup> noted in *ad libitum* feeding experiments using young growing rats that a progressive increase in the level of dietary fat resulted in an apparent increase in the efficiency of protein



utilization when the protein per calorie ratio was kept constant. Since food intake varies inversely with food energy concentration, protein intake would decrease as the percentage of dietary fat is increased. The limitation imposed by this factor on interpretation of data from *ad libitum* feeding experiments is, therefore, easily appreciated. In continuation of the latter experiments, Yoshida, Harper and Elvehjem<sup>4</sup> observed that the growth rate of rats fed on diets containing different levels of fat, but having the same protein per calorie ratio, increased when the level of dietary fat was raised from 0 to 30 per cent. The gain per calorie was also greater when the fat level was raised, the effect being particularly evident during the first week of the experiment. Nitrogen balance studies indicated that the greater rate of gain and improved calorie utilization were not attributable to an improvement in protein utilization, but rather to an apparently more efficient calorie utilization in relation to N intake. The authors concluded that calorie utilization and nitrogen retention in the growing rat are not affected appreciably by the fat content of the diet, but that protein to calorie ratio is an important factor to be considered in interpreting such measurements.

Of particular interest to us was the observation of Forbes and his associates<sup>23</sup> that N retention on low fat diets is improved when the supply of certain vitamins viz., carotene, thiamine, riboflavin, pyridoxine, niacin, pantothenic acid, choline  $\alpha$ -tocopherol, p-aminobenzoic acid and 2, methyl-1, 4 naphthoquinone, in the diets is greatly augmented. In a previous communication from this laboratory,<sup>24</sup> it was demonstrated that, in the rat, B vitamins administered at optimal levels counteract the deterioration in N retention arising from split feeding of the protein and carbohydrate moieties of the diet.<sup>25, 26</sup> It was, therefore of interest to examine the influence of dietary levels of certain B vitamins on protein utilization in the rat from diets with constant or varied protein per calorie (prot./cal.) and with altered levels of dietary fat.

## Experimental

Male weanling albino rats of the Wistar strain were used, with 8 rats per group. The average initial weight of the rats was 45 g. The sources of protein and fat were casein and peanut oil respectively, while corn starch and sucrose, in the proportion 5:1, served as the carbohydrate source. All diets contained 4 per cent of salt mixture (U. S. P. XIV) and the following amounts of water soluble vitamins (in milligrams per kilogram of diet): thiamine HCl, 16; riboflavin, 12; pyridoxine HCl, 8; niacin, 100; inositol, 400; and biotin, 0.8. The above amounts of water soluble vitamins have been considered as adequate at the elevated levels of dietary fat<sup>4</sup>. The quantities of pantothenic acid, choline, vitamin B<sub>12</sub> and folic acid supplied by the diets varied according to the experimental design. The amounts of fat soluble vitamins per rat per week were: vitamin A, 2000 I. U.; vitamin D<sub>2</sub>, 150 I. U.;  $\alpha$ -tocopherol, 10 mg and menadione 1.2 mg. These were administered orally with three drops of peanut oil.

In experiment 1, all four diets contained 10% of protein and four different levels of fat (5, 10, 15 and 20%) and the following marginal levels of the remaining water soluble vitamins (in milligrams per kilogram of diet): calcium pantothenate, 10; folic acid, 1 and choline HCl, 100. No vitamin B<sub>12</sub> was provided as the intestinal synthesis of the vitamin could be assumed to provide the marginal needs. Each of these four basal diets was then modified as follows: diet A supplied 100mg of calcium pantothenate; diet B supplied 0.2 mg of vitamin B<sub>12</sub>; diet C supplied 10 mg of folic acid; diet D supplied both



vitamin B<sub>12</sub> (0.2 mg) and folic acid (10 mg); and diet E supplied a mixture of choline HCl (5 g) and methionine (5 g) per kilogram of diet. Thus, at each level of dietary fat there were six different groups.

In experiment 2, there were 3 series of diets, each having a different Prot./Cal. ratio. Within each series there were three groups which received 5, 10 or 20 per cent of fat. The water soluble vitamins in these diets now included calcium pantothenate (100 mg per kg of diet), choline HCl (5gm per kg of diet) and folic acid (1 mg per kg of diet). Within each series and corresponding to each group there were groups receiving 10 mg of folic acid and 0.2 mg of vitamin B<sub>12</sub> per kilogram of diet. Thus, there were six different groups within each series.

All experiments were of four weeks' duration. The rats were fed *ad libitum*. Food consumption and the nitrogen content of urine and feces were determined for the last three days of each week. The caloric content of each diet was calculated on the basis of the following values<sup>27</sup>; casein, 3.90 Cal. ( $4.55 \text{ Cal} \times \text{N per cent}/100 \times 6.25$ ); peanut oil, 9.45 Cal; starch 4.22 Cal; and sucrose, 3.96 Cal. The nitrogen content of each diet was estimated by the Kjeldahl procedure. Stocks of experimental diets were stored at 4° C.

## Results

*Effects of pantothenic acid, folic acid, vitamin B<sub>12</sub>, choline and methionine:* In experiment 1 (Fig. 1 and 2), the protein per calorie ratios were 24.6, 23.0, 21.6 and 20.4 for the diets containing 5 per cent 10 per cent, 15 per cent, and 20 per cent fat respectively, all four diets containing the same amount of protein (10 per cent).

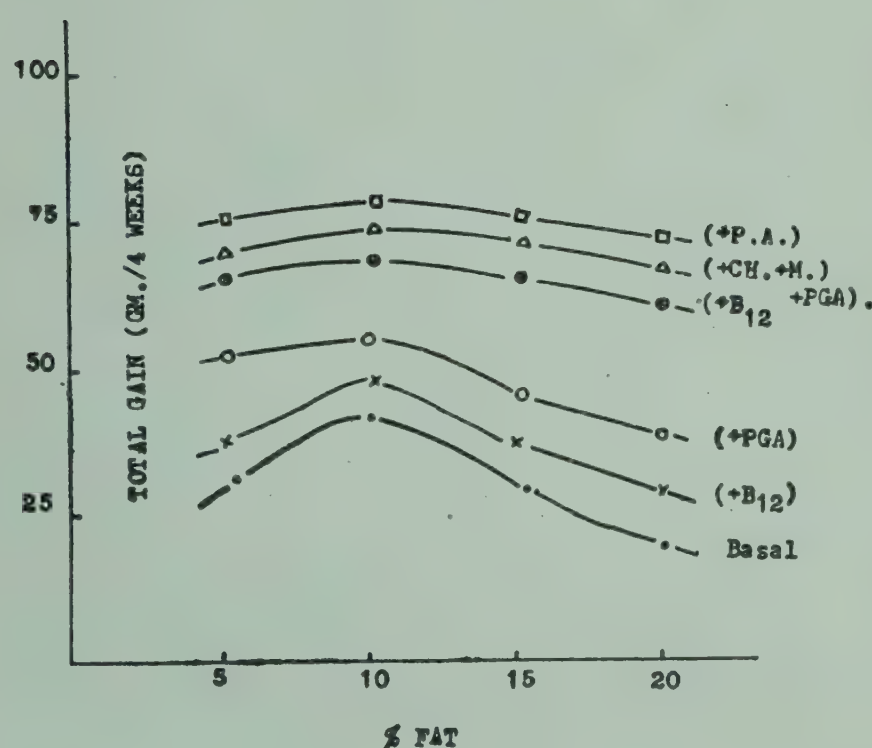


FIG. 1

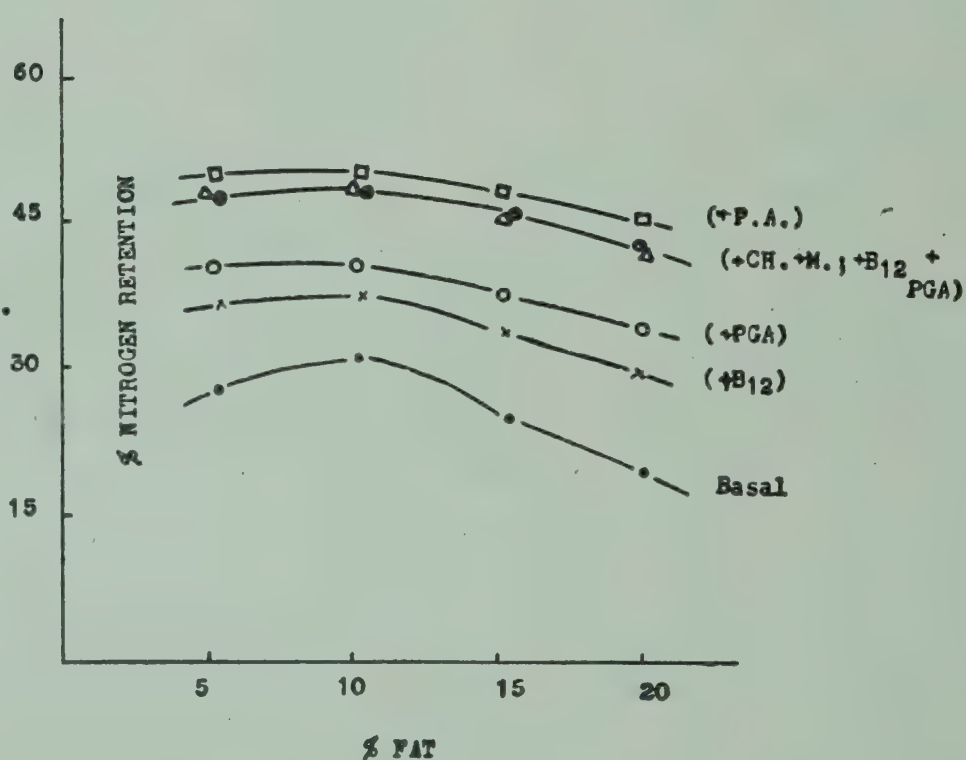


FIG. 2

Effects of pantothenic acid (PA), Choline (CH), Methionine (M), Vitamin B<sub>12</sub> (B<sub>12</sub>) and folic acid (PGA)

In the basal diet series, the group receiving the 10 per cent diet showed the greatest body gain during the four-week period. On both high and low fat containing diets the growth rate was appreciably reduced; the reductions were more with 20 per cent than with 15 per cent fat intake. These differences tended to level off and the growth rate



markedly improved when the dietary supply was augmented with pantothenic acid, or choline and methionine or folic acid or vitamin B<sub>12</sub>. The effectiveness of folic acid and vitamin B<sub>12</sub> was enhanced when these were given together, their joint effect being comparable to that of the mixture of choline and methionine. Pantothenic acid was apparently the most effective of the B vitamins studied.

In the basal diet groups, the percentage nitrogen retention was highest (32 per cent) with 10 per cent fat intake but the nitrogen retention of the group receiving the 5 per cent fat diet was nearly the same (27.5 per cent). The effects of supplemental vitamins and methionine were almost similar to those marked on the growth rate. The effects due to choline and methionine alienated those due to vitamin B<sub>12</sub> and folic acid and vitamin B<sub>12</sub> and folic acid as single supplements had similar effects.

*Effects of vitamin B<sub>12</sub> and folic acid:* In animals on marginal intakes of folic acid and vitamin B<sub>12</sub>, in experiment 2, there was a gradual retardation of the growth rate with increasing amounts of protein per calorie, although the differences were not marked (Figure 3). Thus the four-week gains were 64 g with about 20 mg of Prot./Cal. and 54 g with about 40 mg of Prot./Cal. There was no difference in the growth rate due to fat level at a constant Prot./Cal. ratio. With increased intake of folic acid and vitamin B<sub>12</sub> the growth rate was accelerated, and the gains increased as the amount of protein per calorie was increased. The four-week gains were between 82 and 95 g with about 20 mg of Prot./Cal.; these rose to 110 to 138 g with about 40 mg. of Prot./Cal. Throughout the whole range of Prot./Cal. the gains were greater when the fat content of diet was raised.

The percentage nitrogen retention in animals on low-vitamin diets was fairly constant as the Prot./Cal. ratio shifted from about 20 mg to 30 mg and was between 55 and 46 per cent. However, with about 40 mg of Prot./Cal. the values were lower (34 to 41 per cent) (Fig. 4). At constant Prot./Cal. the nitrogen retention decreased when the fat level was raised. With augmented supply of vitamin B<sub>12</sub> and folic acid, the nitrogen retention improved markedly in all the groups. With intakes of about 20 mg of protein per calorie the,

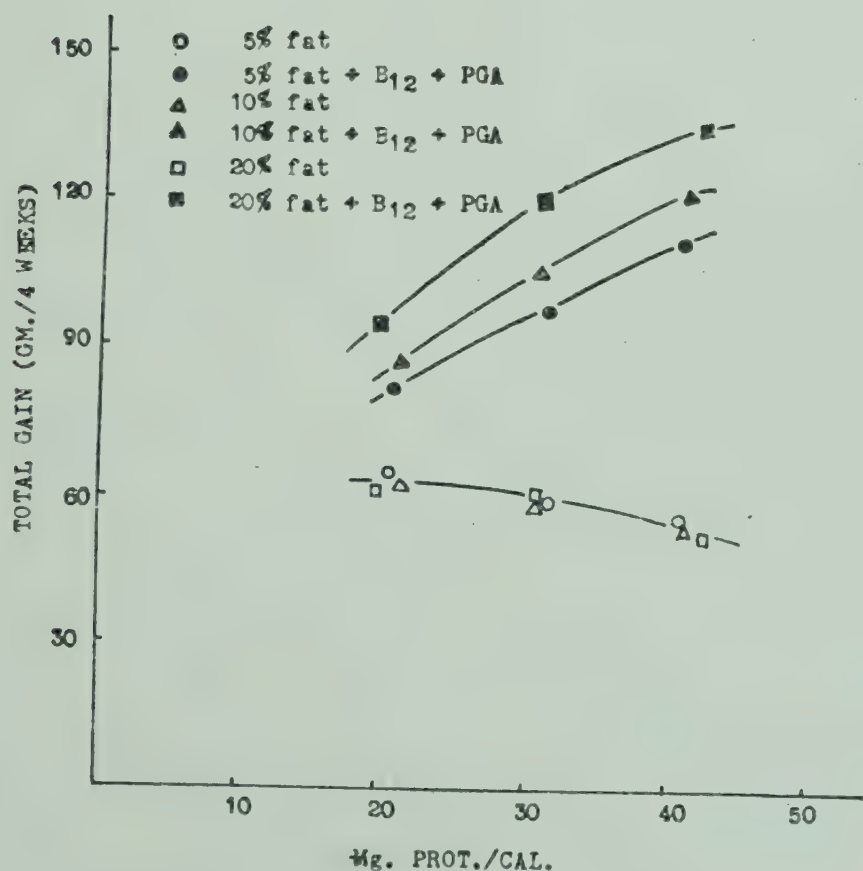


FIG. 3

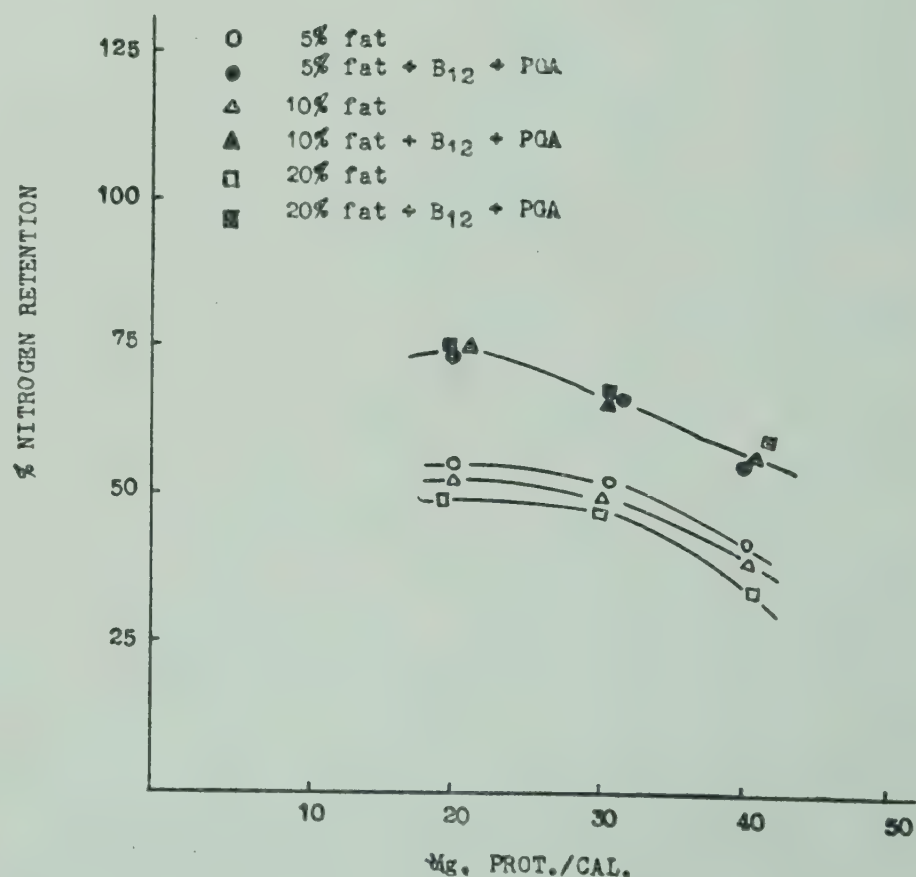


FIG. 4

Effects of vitamin B<sub>12</sub> (B<sub>12</sub>) and folic acid (PGA).



nitrogen retention was 75 per cent. This fell to 68 per cent with about 30 mg of Prot./Cal. and further to 58 per cent with about 40 mg of Prot./Cal. No differences existed due to fat level at a constant Prot./Cal.

## Discussion

The present observations are in agreement with those of Yoshida and coworkers<sup>4</sup> when groups receiving higher supplements of B vitamins in the present study are compared with the growth and N retention curves obtained by the latter workers under somewhat identical experimental conditions. In fact, the observations of the Wisconsin group on the effects of protein per calorie ratio and dietary level of fat on calorie and protein utilization have served as the basis for evaluating the effects of supplemental B vitamins and methionine in the present study.

The results of experiment 1 indicate that in animals on marginal intakes of vitamins both growth rate and nitrogen retention are maximum at 10 per cent fat level than either at 5 per cent or at 15 and 20 per cent fat levels, the protein content being constant. These effects are understandable since, as the content of fat in a diet is increased while the level of protein is kept constant, the Prot./Cal. ratio is decreased and the protein intake is reduced<sup>4</sup>. If protein is limiting for growth, an increase in the level of dietary fat will then depress the growth rate. However, the percentage nitrogen retention may not be affected under these conditions as was also demonstrated by Bosshardt *et al.*<sup>28</sup>, Seller *et al.*<sup>29</sup>, Yoshida *et al.*<sup>4</sup> and others. Similarly, at 5 per cent fat intake perhaps a greater proportion of protein is used for energy purposes, which may affect the growth rate but not the percentage nitrogen retention. Our observations on N retention, especially with high fat diets, are to the contrary. However, the diets employed by us were also limiting with respect to certain B vitamins. Supplements of these vitamins and methionine not only caused general improvements in the growth rate and nitrogen retention but also levelled off differences in these caused by higher or lower fat diets. These results would point to an effect of B vitamins, which is, besides their general effect on growth and nitrogen retention, specifically of moderating nitrogen utilization at altered caloric intakes. Forbes and associates have observed similar improvements in N retention on low fat intakes when the over-all vitamins supply in their diets was augmented.

The effects due to vitamin B<sub>12</sub> and folic acid are probably related, at least in part, to their lipotropic functions as similar effects were also obtained with a mixture of choline and methionine, and since efficient utilization of fats for caloric purposes would involve adequate availability of lipotropic factors. It is also possible that in a diet with high proportion of fat the requirement for pantothenic acid is increased.

These effects are more strikingly brought out in experiment 2 where, with marginal intakes of vitamin B<sub>12</sub> and folic acid, there was complete retardation of the growth rate as the Prot./Cal. ratio increased with apparently no differences due to fat content of the diet. These results are as would be expected, if vitamin B<sub>12</sub> and folic acid influenced not only nitrogen utilization but also calorie utilization.

With optimal intakes of the vitamins, the improved growth rate with higher Prot./Cal. is attributable to an increase in protein intake, while at a constant Prot./Cal. higher gains on high-fat diets are probably due to a higher caloric intake (and efficient caloric utilisation) while the protein intake remained proportionately constant.



Sibbald *et al.*<sup>30</sup> using a mixture of casein, lactalbumin, DL-methionine, L-histidine and DL-threonine as the protein source indicated that percentage of nitrogen retention gradually decreased as the Prot./Cal. was increased from 21 to 47 mg. On the other hand, Yoshida *et al.*<sup>4</sup>, using casein as the sole protein source, found a fairly constant nitrogen retention over the same range. The latter group attributed this discrepancy to the lower biological value of casein which would call for a higher protein intake to satisfy the protein requirement. Since nitrogen intake increases as the Prot./Cal. is increased, percentage of nitrogen retention would decrease once this point was exceeded. This would occur at lower Prot./Cal. ratios with a protein of high biological value. In our experiments, the percentage N retention remained fairly constant at marginal intakes of vitamins B<sub>12</sub> and folic acid within the range of 20 to 30 mg. of protein per calorie. However, when the diet was fortified with vitamin B<sub>12</sub> and folic acid drop in N retention within this range was relatively sharp and continued till 40 mg. of Prot./Cal., although there were general increments in N retention due to the vitamins. This could probably be interpreted as indicating an improvement in the biological value of casein with higher supplements of the vitamins.

At the same Prot./Cal. ratio the percentage N retention was not affected by the fat content of the diet when this supplied adequate levels of the vitamins. This is in agreement with the observations of Forbes *et al.*,<sup>23,31</sup> French *et al.*<sup>32</sup> and Yoshida *et al.*<sup>4</sup> and suggests that fat has no effect on protein utilization. However, this apparently is also dependent upon the adequacy of the diet with respect to vitamin B<sub>12</sub> and folic acid (and possibly also other B factors) as is evident from the lowered N retention with marginal intakes of the vitamins when the fat content of the diet is raised.

In conclusion, it may be said that, in addition to protein intake and the ratio of protein to calories, the B vitamins status is an important factor affecting both calorie and nitrogen utilization.

The beneficial effects of B vitamins supplied at optimal levels on N utilization from diets in which protein and caloric components are fed at an interval have already been demonstrated<sup>24</sup>. It is possible that B vitamins influence a more balanced selection of food components by the animal. Richter and Hawkes<sup>33</sup> showed, for example, that rats select varying proportions of protein, fat and carbohydrate when supplied with different B vitamins, presumably adjusting the intake to actual needs. And there is also probably a good deal of flexibility in nutritional needs based upon the stresses to which the organism is subjected<sup>34</sup>.

## Summary

The effects of changes in the protein per calorie ratio of the diet and in the level of dietary fat on growth and nitrogen retention have been examined in relation to dietary levels of folic acid, vitamin B<sub>12</sub>, pantothenic acid, choline and methionine using young rats fed on purified diets containing casein as the sole source of protein.

In 10 per cent protein diets with marginal provision of folic acid, vitamin B<sub>12</sub>, pantothenic acid and choline, gains in weight and percentage nitrogen retention were maximal at 10 per cent fat level (Prot./Cal.-23 mg) and reduced with both increasing (15 and 20 per cent) or decreasing (5 per cent) fat levels. These differences considerably levelled off, and growth rate and nitrogen retention improved, when the diet was fortified to contain



10 mg per cent of pantothenic acid, or 0.5 per cent of choline and 0.5 per cent of methionine, or 20  $\mu$ g per cent of vitamin B<sub>12</sub> and 1 mg per cent of folic acid, in decreasing order of efficacy. The efficacies of vitamin B<sub>12</sub> and folic acid were improved when these were administered together.

In another experiment, animals on marginal intakes of folic acid and vitamin B<sub>12</sub> showed retardation in growth rate as the amount of protein per calorie in the diet was increased, there being no changes due to variations in fat content of the diet at a constant Prot./Cal. ratio. The percentage N retention remained fairly constant over a range of 20 to 30 mg of Prot./Cal., but decreased as this ratio was shifted to 40 mg. At constant Prot./Cal. ratio an increase in fat level caused somewhat reduced N retention. Augmented levels of vitamin B<sub>12</sub> and folic acid caused general increments in the growth rate and in N retention and improved calorie utilization.

It is concluded that, apart from protein intake and the ratio of protein to calories, the B vitamins status of the animal is an important factor affecting both calorie and protein utilization.

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# A STUDY OF THE EFFECT OF VARIATION IN DIETARY FAT LEVELS, PROTEIN QUALITY AND B VITAMINS SUPPLEMENTATION, ON LIPID COMPOSITION AND DISTRIBUTION IN BLOOD AND LIVER IN RATS\*

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The food intake of weanling rats is markedly influenced by the available energy content of the diet. It has been suggested<sup>1</sup> that, within physiological limits, animals eat to satisfy their energy requirements. Hill and Dansky<sup>2</sup> observed that food intake was chiefly influenced by the energy content of the ration, while the protein level has little or no effect. The ratio of protein to available energy exerts a considerable influence on fat deposition in animals<sup>3</sup>. Dietary fat has a protein sparing action<sup>4</sup> which may be influenced by the calories: protein ratio. It has been observed<sup>5</sup> that optimum growth and optimum feed efficiency are closely related to a rather sensitive balance between the amount of protein and the caloric content of the diet. Thus, the growth promoting potential of an adequate diet cannot be based on the percentage of protein alone, but the calories: protein ratio has also to be considered.

In recent years, a great deal of evidence has accumulated<sup>6</sup> implicating dietary fat in the causation of cardiovascular disease. Interest has chiefly centred round the relationship of dietary fatty acid components to the metabolism of cholesterol. More recent work implicates dietary proteins<sup>7, 8</sup> and vitamins<sup>6, 9</sup> as well. In view of the above, it was thought to be of interest to study the effects of variations in calories: protein ratio on lipid composition and distribution in blood and liver under conditions varied with respect to B vitamins intake.

## Experimental

Male weanling albino rats (laboratory bred, Wistar strain) weighing about 45 g were used. They were grouped and fed as given Table I. Data on growth and food intake were recorded during the experimental period of 16 weeks, at the end of which the animals were sacrificed under ether anaesthesia, and blood and liver samples collected for analysis. Blood was withdrawn from the portal vein, immediately heparinized and centrifuged in the cold to obtain the plasma. Livers were quickly removed and cooled in crushed ice. They were separated from adhering connective tissue, weighed and 20 per cent homogenates in ice-cold water were prepared using a Potter-Elvehjem glass homogeniser. Aliquots of the homogenates were used for the various determinations. The analytical procedures adopted were the same as those already described by the authors in a previous paper in this symposium.

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TABLE I

*Effect of variations in protein quality, fat levels and B vitamins supplementation on growth and food intake*

Group	Diet	80% fat			15% fat		
		Average gain in weight (g) during		Average daily food intake (g)	Average gain in weight (g) during		Average daily food intake (g)
		8 weeks	16 weeks		8 weeks	16 weeks	
I	Casein (18% protein) ...	99.5	188.7	9.1	86.2	171.5	8.3
	+ Minimal B-vitamins ...	± 3.6	± 7.5	± 0.4	± 4.5	± 6.0	± 0.3
II	Casein (18% protein) ...	108.6	206.2	9.6	97.7	187.4	8.7
	+ Optimal B-vitamins ...	± 4.9	± 6.4	± 0.2	± 5.2	± 6.3	± 0.5
III	Wheat-gluten (18% protein) ...	60.8	135.5	7.8	51.4	116.8	6.5
	+ Minimal B-vitamins ...	± 2.8	± 6.2	± 0.4	± 2.7	± 5.5	± 0.4
IV	Wheat-gluten (18% protein) ...	75.3	162.4	8.4	68.6	141.3	7.3
	+ Optimal B-vitamins ...	± 2.6	± 7.1	± 0.5	± 3.1	± 5.7	± 0.3

Male, weanling, albino rats (Wistar strain, laboratory bred) were divided into 8 groups and fed *ad libitum* for 16 weeks on 18 per cent protein (case in or wheat-gluten (N.B.C.) ) diet supplemented with hydrogenated vegetable oil (Dalda) at 8 and 15 per cent levels and varied with respect to certain of the B vitamins at minimal and optimal levels as given in Table II. Other additions were (per kg. diet): salt mixture, U.S.P., XIV, 40 g; vitamin A 5000 I.U.;  $\alpha$ -tocopherol 50 mg; vitamin K (menadione), 10 mg; and choline chloride 500 mg.

Results are averages of four independent samples  $\pm$  S.E.M.

## Results and Discussion

Data on growth and food intake are summarized in Table II. On the high fat diets, growth rate was impaired, presumably due to the decreased food intake. The reduced food intake is, however, not surprising in view of the fact that animals of a given species eat to satisfy their calorie requirements<sup>1, 10, 11</sup>. While the level of intake of B vitamins did not appear to affect the impairment in growth rate, at the higher level of intake of B vitamins an improvement in growth was observed and the difference in growth due to protein quality was reduced.

These results are corroborated by the results obtained for liver and plasma total nitrogen (Table III). On the high fat diets, total nitrogen was reduced but was improved in presence of higher levels of B vitamins. While liver non-protein nitrogen was not affected, higher levels of B vitamins decreased plasma non-protein nitrogen. Total lipids in liver and plasma (Table IV) were markedly increased on the high fat diet. No significant alteration due to levels of B vitamins was observed.

Liver phospholipids were more or less unchanged, but in plasma, reduced levels of phospholipids were obtained on the wheat gluten diet. Increased provision of B vitamins raised the levels in both the casein and wheat gluten diets (Table IV).

As expected, there was an increase in liver and plasma cholesterol (Table V) on the high fat diet. This increase was chiefly due to the increase in ester cholesterol as can be



TABLE II

*Levels of B vitamins added to the diet*

Vitamins		mg/100 g diet	
		Optimal	Minimal
Thiamine hydrochloride	...	0.300	0.075
Riboflavin	...	0.400	0.100
Pyridoxine hydrochloride	...	0.300	0.075
Calcium-d pantothenate	...	1.000	0.250
Nicotinic acid	...	2.000	0.500
Vitamin B <sub>12</sub>	...	0.015	nil
Folic acid	...	0.100	0.025

Inositol 50.0 mg., p-amino benzoic acid 10.0 mg. and biotin 0.10 mg. per 100 g. diet were added at the same levels.

TABLE III

*Changes in liver and plasma nitrogen*

Group	8% Fat				15% Fat			
	Liver		Plasma		Liver		Plasma	
	Total nitrogen	Non-protein nitrogen	Total nitrogen	Non-protein nitrogen	Total nitrogen	Non-protein nitrogen	Total nitrogen	Non-protein nitrogen
	mg/g fresh wt.		mg/100 ml		mg/g fresh wt.		mg/100 ml	
I	32.2 ± 1.8	2.2 ± 0.06	1095 ± 26	45.0 ± 1.8	28.4 ± 0.9	2.6 ± 0.11	920 ± 18	47.3 ± 1.6
II	35.9 ± 1.4	2.7 ± 0.26	1330 ± 34	40.0 ± 2.1	31.6 ± 1.5	2.3 ± 0.08	1125 ± 31	42.0 ± 2.0
III	26.9 ± 0.7	2.5 ± 0.19	887 ± 21	52.0 ± 3.2	22.1 ± 1.1	2.5 ± 0.13	712 ± 27	50.0 ± 4.0
IV	31.4 ± 1.3	2.8 ± 0.4	1006 ± 43	47.0 ± 3.0	25.6 ± 0.8	2.2 ± 0.05	854 ± 32	45.0 ± 1.7

For details refer footnote Table I.



TABLE IV  
Changes in liver and plasma lipids

Group	8% Fat				15% Fat			
	Liver		Plasma		Liver		Plasma	
	Total lipids	Phospho-lipids	Total lipids	Phospho-lipids	Total lipids	Phospho-lipids	Total lipids	Phospho-lipids
	mg/g fresh wt.		mg/100 ml		mg/g fresh wt.		mg/100 ml	
I	61.3 ± 1.9	22.6 ± 0.8	302 ± 28	123.1 ± 7.7	76.6 ± 2.8	23.3 ± 2.0	340 ± 27	131.2 ± 7.3
II	63.4 ± 2.2	26.7 ± 1.2	296 ± 24	151.5 ± 8.2	74.2 ± 1.6	25.6 ± 1.4	334 ± 32	145.8 ± 8.6
III	60.0 ± 1.7	19.5 ± 0.8	290 ± 15	97.9 ± 5.3	80.2 ± 3.4	20.1 ± 1.0	326 ± 26	97.9 ± 9.0
IV	62.1 ± 0.8	24.5 ± 1.6	285 ± 13	126.5 ± 9.5	78.8 ± 2.4	23.3 ± 1.8	318 ± 30	104.1 ± 4.7

For details refer footnote Table I

TABLE V  
Changes in liver and plasma cholesterol

Group	8% Fat				15% Fat			
	Liver		Plasma		Liver		Plasma	
	Free	Total	Free	Total	Free	Total	Free	Total
	mg/100 g fresh wt.		mg/100 ml		mg/100 g fresh wt.		mg/100 ml	
I	214 ± 9	436 ± 12	22 ± 1.2	98 ± 2.6	292 ± 6	642 ± 17	31 ± 1.8	156 ± 4.6
II	221 ± 7	430 ± 14	19 ± 0.6	92 ± 3.1	278 ± 9	651 ± 15	28 ± 1.2	144 ± 5.1
III	185 ± 4	320 ± 11	17 ± 0.4	66 ± 1.5	235 ± 7	408 ± 12	22 ± 0.8	96 ± 3.1
IV	178 ± 8	312 ± 8	15 ± 0.7	59 ± 1.9	216 ± 5	386 ± 13	20 ± 0.6	90 ± 3.3

For details refer footnote Table I

seen from the results. In presence of higher levels of B vitamins, the cholesterol levels were not significantly altered. However, a distinct reduction in the levels was observed in the wheat gluten groups at both levels of fat intake. Such an effect of protein quality has also been observed by others<sup>12, 15</sup>. Nath *et al.*<sup>12</sup>, comparing the effects of various dietary levels of protein of both animal and vegetable origin on serum cholesterol levels in the



rat, observed that the serum cholesterol values on wheat gluten diets were lower than values on casein diets at each level of dietary protein at which comparison was made.

Essentially all lipids in plasma occur as lipoprotein complexes<sup>14</sup> and the  $\beta$ -lipoprotein components account for most of the lipid variation. The data on changes in concentration of total lipid, total cholesterol and phospholipids in  $\alpha$ - and  $\beta$ -lipoproteins (Tables VI and VIII) bring out some interesting observations. While the concentration of total lipids in the lipoprotein fractions increased on the high fat diets, the percentage in the  $\beta$ -lipoprotein fraction remained almost constant. There did not appear to be much change due to vitamin intake or protein quality.

TABLE VI  
*Total lipids in  $\alpha$ - and  $\beta$ -lipoproteins of plasma*

Group	8% Fat			15% Fat		
	$\alpha$ -	$\beta$ -	%in $\beta$ -portion	$\alpha$ -	$\beta$ -	%in $\beta$ -portion
	mg/100 ml of plasma			mg/100 ml of plasma		
I	121 $\pm 3.2$	166 $\pm 4.4$	57.8	145 $\pm 3.6$	173 $\pm 5.3$	54.4
II	124 $\pm 2.4$	152 $\pm 3.5$	55.9	144 $\pm 2.5$	168 $\pm 3.8$	53.8
III	113 $\pm 4.1$	160 $\pm 4.7$	58.3	134 $\pm 1.9$	175 $\pm 4.2$	56.6
IV	116 $\pm 3.7$	153 $\pm 2.9$	56.7	143 $\pm 4.2$	164 $\pm 4.0$	53.2

For details refer footnote Table I

TABLE VII  
*Phospholipids in  $\alpha$ - and  $\beta$ - lipoproteins of plasma*

Group	8% Fat			15% Fat		
	$\alpha$ -	$\beta$ -	%in $\beta$ -portion	$\alpha$ -	$\beta$ -	%in $\beta$ -portion
	mg/100 ml of plasma			mg/100 ml of Plasma		
I	44 $\pm 2.7$	84 $\pm 3.5$	64.9	38 $\pm 1.7$	82 $\pm 3.6$	67.6
II	57 $\pm 2.1$	100 $\pm 4.4$	63.2	44 $\pm 2.1$	104 $\pm 4.8$	66.3
III	51 $\pm 1.5$	58 $\pm 2.6$	52.6	46 $\pm 2.4$	56 $\pm 1.6$	54.8
IV	61 $\pm 3.2$	63 $\pm 2.9$	50.8	54 $\pm 3.1$	63 $\pm 2.4$	53.5

For details refer footnote Table I



There was a slight increase in the percentage of phospholipids in the  $\beta$ -fraction on the 15 per cent fat diet. The rather high values obtained may probably be due to the prolonged feeding of hydrogenated fat which could have caused a derangement in fat metabolism. On feeding the wheat gluten diets, however, there was a marked decrease in the phospholipid concentration in the  $\beta$ -fraction tending to bring it within the normal limits.

Approximately two-thirds of plasma cholesterol is generally found in the  $\beta$ -lipoprotein of plasma. As may be seen from the results (Table VIII), there is an increased concentration of cholesterol in the  $\beta$ -fraction on the 15 per cent fat diet. A reduction due to wheat gluten is obtained which can be more clearly observed on the 15 per cent fat diet. These results are thus consistent with the results obtained for cholesterol in whole plasma.

TABLE VIII  
Total cholesterol in  $\alpha$ - and  $\beta$ -lipoproteins of plasma

Group	8% Fat			15% Fat		
	$\alpha$ -	$\beta$ -	%in $\beta$ -portion	$\alpha$ -	$\beta$ -	%in $\beta$ -portion
	mg/100 ml of plasma			mg/100 ml of plasma		
I	26 $\pm 0.6$	66 $\pm 1.3$	70.8	32 $\pm 1.1$	112 $\pm 3.5$	77.6
II	27 $\pm 1.3$	56 $\pm 2.1$	68.5	30 $\pm 1.4$	105 $\pm 2.4$	77.2
III	21 $\pm 0.5$	41 $\pm 1.4$	65.8	29 $\pm 0.6$	62 $\pm 3.1$	67.6
IV	20 $\pm 0.9$	34 $\pm 0.8$	63.9	27 $\pm 0.5$	56 $\pm 2.7$	66.4

For details refer footnote Table I

Summary

The effect of variations in calories: protein ratio on lipid composition and distribution in blood and liver was examined with an 18 per cent protein (casein or wheat-gluten) diet supplemented with fat at 8 and 15 per cent levels and varied with respect to certain of the B vitamins at minimal and optimal levels.

On the high fat diets, food intake was reduced and growth rate was impaired, irrespective of the level of intake of B vitamins. Total nitrogen in liver and plasma were reduced on the high fat diets while liver non-protein nitrogen was not altered. In presence of higher levels of B vitamins, liver and plasma nitrogen contents were improved.

Total lipids, and total cholesterol in liver and plasma were increased on the 15 per cent fat diets. No effect of supplementation with higher levels of  $\beta$  vitamins could be observed. Total phospholipids were somewhat reduced on the wheat gluten diet.

Total lipids, total cholesterol and phospholipids in the  $\alpha$ - and  $\beta$ -lipoprotein fractions were studied. While the percentage of total lipids in the  $\beta$ -lipoprotein fraction was steady,



there was a slight increase in phospholipid percentage on the 15 per cent fat diet. On this diet there was an enhanced concentration of cholesterol in  $\beta$ -lipoprotein fraction which was reduced on the wheat-gluten diet.

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# THE RELATIONSHIP OF DIETARY B VITAMINS AND PROTEINS TO REGENERATION OF TISSUE CONSTITUENTS IN THE PROTEIN DEPLETED RAT\*

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It has been reported that in protein-fasted adult rats, the level of intake of B vitamins may influence protein utilization from diets with uneven distribution of protein and caloric moieties or with intermittent changing levels of protein<sup>1</sup> as well as from single or mixed protein diets<sup>2</sup>. Work from this laboratory has also indicated the beneficial effect of B vitamins in the utilization of low quality protein dietaries. The present report relates to observations on the changes in certain liver and plasma constituents resulting from protein starvation in young adult rats and on the influence of dietary levels of certain B-vitamins (thiamine, riboflavine, niacin, pantothenic acid, pyridoxine, folic acid and vitamin B<sub>12</sub>) on the rate of regeneration during protein realimentation. The effects of protein quality and quantity as well as specifically of dietary folic acid and vitamin B<sub>12</sub> have also been examined in relation to tissue constituents in protein-fasted and refed rats.

A deficiency in dietary nitrogen results in an imbalance in protein stores and is characterized by a shift in the distribution of blood and tissue proteins. The pattern of enzyme systems is altered markedly in the liver and to a lesser degree in other tissues. A shift in metabolism involving all nutrients is associated with a loss in tissue proteins. The protein stores can be regenerated by refeeding of proteins, the rate and extent of such repletion being obviously determined by the quality and quantity of the fed protein as well as other dietary essentials including vitamins and minerals.

## Experimental Methods

Livers were removed from the freshly killed animals and 10 per cent homogenates were prepared in isotonic sucrose (0.25 M)-CaCl<sub>2</sub> (0.0018M) solution<sup>3</sup> using a Potter-Elvehjem type glass homogeniser. Fractionation of the homogenate (15 ml) was accomplished by the procedure of Schneider and Hogeboom<sup>4</sup> using an International Refrigerated Centrifuge (PR-2). The nuclei were sedimented at  $700 \times g$  for 10 minutes and resuspended in 15 ml cold isotonic sucrose solution after once washing and used as the nuclear fraction. The supernatant from the first centrifugation and the washings were mixed together and spun at  $5000 \times g$  for 10 minutes. The mitochondrial pellet was washed once and then resuspended in 15 ml sucrose solution, and used as the mitochondrial fraction. The combined unsedimented supernatant and the mitochondrial washings were labelled as the supernatant consisting of microsomes and the soluble portion of the liver cell.

Nitrogen in whole homogenates and in the centrifugally separated fractions and in plasma was determined by a micro-Kjeldahl procedure<sup>5</sup>.

Activities of xanthine oxidase by the method of Dhungat and Sreenivasan<sup>6</sup> and succinoxidase according to Schneider and Potter<sup>7</sup> and of malic dehydrogenase<sup>8</sup> were followed

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manometrically in Warburg vessels. Alkaline phosphatase activity in nuclear fraction was determined in a Palade's system<sup>9</sup> and the liberated phosphate was determined according to the method of Taussky and Shorr<sup>10</sup>. The ability of liver to convert folic acid (PGA) to citrovorum factor (CF) was carried out under the conditions reported optimal<sup>11, 12</sup>. CF was assayed with *Leuconostoc citrovorum* ATCC 8081 (since identified as *Pediococcus cerevisiae*) using leucovorin (calcium) (*Lederle*) for standard with basal medium of Sauberlich and Baumann<sup>13</sup>.

Free and total riboflavin and flavin adenine dinucleotide (FAD) were determined by the fluorimetric method of Bessey *et al.*<sup>14</sup>. Differential determination of pyridine nucleotides (PN) as oxidized and reduced forms was carried out according to the method of Dianzani<sup>15</sup>. Coenzyme A (CoA) was determined by the procedure of Kaplan and Lipmann<sup>16</sup>. Cocarboxylase (TPP) expressed as the difference between free and total thiamine was determined fluorimetrically by the method of Conner and Straub<sup>17</sup>.

Livers were also assayed microbiologically for pantothenic acid using *Lactobacillus arabinosus* (ATCC 8014) with the medium of Skeggs Wright<sup>18</sup> and folic acid using *Streptococcus faecalis* R (ATCC 8043) with the medium of Mitbander and Sreenivasan<sup>19</sup> and colorimetrically for nicotinic acid<sup>20</sup>.

Total liver non-protein sulfhydryl (SH) was determined according to Grunert and Phillips<sup>21</sup>. Nucleic acids were extracted from liver tissue according to Schneider<sup>22</sup>, ribonucleic acid (RNA) was estimated by a modification<sup>23</sup> of the orcinol method<sup>24</sup> and desoxyribonucleic acid (DNA) by the diphenylamine colour reaction of Dische<sup>25</sup>.

Arginine in nuclear fraction was estimated microbiologically with *streptococcus faecalis-R*<sup>26</sup>, samples were prepared by hydrolysing aliquots of the fraction in 6N hydrochloric acid for 10 hours in sealed bulbs at 10 lbs/sq. in. steam pressure.

Total serum protein was determined on 30  $\mu$ l portions by the biuret method<sup>27</sup>. Fractionation of protein was carried out by electrophoresis of the serum on Whatman No. 3 paper strips using barbiturate buffer of pH 8.6 and ionic strength 0.075. Separation was effected at a constant current of 2.5 mA per strip in a horizontal open strip type cell for 16 hours at room temperature (28°). Strips were stained with bromophenol blue as described by Jencks *et al.*<sup>28</sup>. The relative concentrations of the protein fractions were read densitometrically on an 'EEL' scanner (Evans Electroselenium Ltd., Harlow, Essex).

## Results and Discussion

1. *Effect of protein fasting on certain liver cell constituents in the rat*: Protein fasting for a period of 14 days causes a loss of nitrogen from all the cytoplasmic fractions and is largest in the microsomal and soluble proteins (Table I). While reductions in succinoxidase and alkaline phosphatase activities amounted to 28 and 23 per cent respectively, there is a total disappearance of xanthine oxidase activity. The reduction in RNA is confined largely to the supernatant and mitochondrial fractions. There is an increase in DNA expressed on unit liver weight. This increase in DNA is only apparent since there is no change in the DNA content of the whole liver and arises from increased cell density<sup>29, 30</sup> due to the loss of labile cytoplasm. That the nuclear protein, known to be a histone, gets depleted during protein fasting could be inferred from the decrease in the proportion of arginine to DNA.



TABLE I  
*Effect of protein fasting (2 weeks) on body weight and liver constituents*

		Control <sup>1</sup> (10 per cent casein)	Protein fasted (2 weeks)
Animal weight (g)	...	120.0 ± 3.7	93.0 ± 2.1
	Homogenate	37.1 ± 1.2	23.4 ± 0.3
	Nuclei	8.8 ± 0.4	6.2 ± 0.6
Liver nitrogen (mg/g. fresh weight)	Mitochondria	7.9 ± 0.4	5.8 ± 0.2
	Supernatant <sup>2</sup>	19.9 ± 1.3	12.1 ± 0.5
Alkaline phosphatase (nuclei) (μgP liberated/hr./g. fresh liver)	...	534 ± 24	122 ± 17
Succinoxidase (mitochondria) (μl O <sub>2</sub> uptake/hr./g. fresh liver)	...	905 ± 26	255 ± 14
Xanthine oxidase (supernatant) (μl O <sub>2</sub> uptake/hr./g. fresh liver)	...	157 ± 11	nil
	Homogenate	1043.4 ± 32.5	665.1 ± 26.2
	Nuclei	268.3 ± 2.4	220.4 ± 2.1
Liver RNA (mg./100g. fresh weight)	Mitochondria	167.2 ± 13.0	76.1 ± 6.2
	Supernatant <sup>2</sup>	695.5 ± 5.3	366.5 ± 16.2
	Homogenate	272.5 ± 19.2	385.2 ± 15.7
	Nuclei	273.5 ± 2.7	383.0 ± 24.6
Liver DNA (mg./100g. fresh weight)	...		
Arginine (nuclei) (mg/g. fresh liver)	...	3.13 ± 0.51	1.32 ± 0.52
Arginine/DNA	...	1.14	0.41

<sup>1</sup> The animals in the control group were maintained on 10 per cent casein diet consisting of (g. per cent): maize starch 70, cellulose 3, sucrose 5, sesame oil 8, and salt mixture (U.S.P. No. XIV) 4. The vitamin additions to the diet were (in mg. per kg. of diet) thiamine hydrochloride 3, riboflavin 4, nicotinic acid 20, calcium pantothenate 10, pyridoxine hydrochloride 3, folic acid 1, vitamin B<sub>12</sub> 0.15, biotin 1, p-aminobenzoic acid 100, choline chloride 500, inositol 500, vitamin K 10 and α-tocopherol 50 and vitamin A 5000 I. U. The protein-free diet was the same as above except that casein was replaced by starch.

Protein starved samples were obtained at the end of 2 weeks of protein fasting.

<sup>2</sup> Supernatant consists of microsomes and the soluble fraction.

The results are the mean from 5 animals ± S.E.



2. *Influence of dietary levels of B vitamins on certain plasma and liver constituents during protein realimentation in the rat:* Young male Wistar rats about 100 g in weight, maintained on a 10 per cent casein diet, were protein-fasted for 2 weeks and refed on 10 per cent casein diet with the two levels of B vitamins, the optimal and minimal, for 2, 4, 7 and 11 days and samples taken at the end of these periods. In another experiment, the animals were starved for one week and repletion studied at the end of 4 and 7 days of refeeding protein.

Protein starvation for 2 weeks appears to be more severe as compared to one week. (Table II). During protein deprivation, in addition to the losses in N and the enzyme activities (succinoxidase, alkaline phosphatase and xanthine oxidase), the liver soluble sulfhydryl content is drastically reduced, the loss from the supernatant fraction being severe and in the nuclear fraction slight. Among the cofactors, PN, FAD and CoA are also depleted during protein fasting. Similar reductions in tissue levels of riboflavin and nicotinic acid have been observed by Seifter *et al.*<sup>32</sup>. This could be due to their existence as protein-bound complexes in the liver. It could also be due to failure of the liver to retain these cofactors. The lowered enzyme activities may thus be a result of loss in cofactors parallel to the loss of protein. The low cofactor content could also be attributed to impaired synthesis of cofactors from the corresponding vitamins and/or depletion of the cofactor specific enzyme protein(s). The possibility may also be considered that the level of

TABLE II

*Regeneration (after 1 week of protein-fasting) on 10 per cent casein diet at 2 levels of B vitamins: growth and liver constituents*

Group	Vitamin level	Animal weight (g)	Liver N mg./g fresh weight	Total SH (supernatant) mg./100 g. fresh liver	$\mu$ l O <sub>2</sub> uptake/hr./g fresh liver		Alkaline phosphatase (particulate) <sup>2</sup> $\mu$ g liberated per hr/g fresh liver	$\mu$ g/g. fresh liver		Co A units per g. fresh liver
					Xanthine oxidase (supernatant)	Succin oxidase (particulate) <sup>2</sup>		FAD	Total PN	
Protein fasted (7 days) ...	Optimal	108.5 ± 2.0	25.7 ± 1.3	13.0 ± 3.8	nil	471 ± 23	333 ± 28	4.8 ± 0.7	689 ± 29	112 ± 3
Control ...	Optimal	129.0 ± 4.0	30.3 ± 0.3	136.0 ± 6.4	99.6 ± 3.7	799 ± 31	702 ± 44	19.6 ± 1.2	900 ± 35	169 ± 7
	Minimal <sup>1</sup>	130.8 ± 3.5	27.4 ± 0.5	62.1 ± 4.8	24.7 ± 2.1	560 ± 22	530 ± 22	9.0 ± 1.2	785 ± 32	141 ± 4
Refed protein (7 days) ...	Optimal	141.5 ± 1.4	28.1 ± 0.6	73.2 ± 4.8	71.8 ± 4.2	598 ± 15	655 ± 26	12.7 ± 1.5	856 ± 18	150 ± 2

<sup>1</sup> The minimal level of the B vitamins in the diet was (in mg. per kg. of diet) thiamine hydrochloride 0.75, riboflavin 1, nicotinic acid 5, calcium pantothenate 2.5, pyridoxine hydrochloride 0.75, folic acid 0.25 and vitamin B<sub>12</sub> nil. The optimal level was the same as given under Table I. The animals were protein-fasted for 1 week and protein realimented for one week.

<sup>2</sup> Fraction sedimenting at 5000 × g.

Other details as under Table I.



certain B vitamins may affect the maintenance and utilization of other vitamins as cofactors. Decreased contents of TPP, FAD and PN in vitamin B<sub>12</sub> deficiency<sup>33, 34</sup> and PN in thiamine deficiency<sup>35</sup> have been reported. Elevated CoA content in B<sub>12</sub> deficiency<sup>36, 37</sup> and that of B<sub>12</sub> in pantothenic acid deficiency<sup>38</sup> have been observed.

Refeeding of protein shows that optimal level of B vitamins promotes faster regeneration of cell constituents. This influence of B vitamins in accelerating the overall regenerative process is undoubtedly related to their widespread participation in metabolism including utilization of dietary amino acids. The cellular contents are partially replenished in animals starved for two weeks. However, with one week protein fasted animals repletion is more marked. It is apparent from these observations, particularly from liver regeneration, that protein fasting for two weeks is somewhat drastic. It has been observed by earlier workers<sup>39</sup> that if refeeding an adequate diet is delayed too long, the rats failed to regain glycogen forming and deaminating mechanisms which may eventually lead to irreversible damage to protein synthesizing processes. The restoration of liver SH is slow and incomplete and the effect due to level of B vitamins is not discernible. The low sulfhydryl content and its slow restoration following protein deprivation could be due to the intrinsic deficiency of sulfur amino acids in the casein diet. Leaf and Neuberger<sup>40</sup> have shown that administration of cystine or methionine restores quickly to normal the low SH levels. Regeneration of the completely depleted xanthine oxidase is slow but B vitamins significantly enhance the rate. Alkaline phosphatase which is lowered during protein depletion is also restored more rapidly in the groups fed the higher level of B vitamins. Similar response is obtained for succinoxidase activity.

Regeneration in PN and CoA approaches that control value with the optimal concentration of B vitamins, while FAD is partially restored.

The serum proteins (Table III) are lowered during protein fasting for two weeks, with concomitant reductions in albumin,  $\alpha_1$ -,  $\alpha_2$ - and  $\beta$ -globulins;  $\gamma$ -globulin, however, remains practically unchanged. These changes in serum protein profile are in accordance with the findings of Allison<sup>41</sup>. The marked reduction of albumin and  $\alpha_1$ -globulin concentration in the protein-fasted animals suggest that the dependence of these fractions on dietary amino acids is greater than of other serum protein fractions. Faster repletion of total serum protein concentration with minimal as compared to optimal intake of B vitamins is seen during protein realimentation. This faster replenishment of serum proteins with minimal than with optimal intake of B vitamins possibly occurs as a result of an impaired utilization of serum proteins for tissue protein synthesis. It appears that this faster repletion occurs through elevation of  $\beta$ - and  $\gamma$ -globulins concentrations with the sub-optimal intake of B vitamins. There is an incomplete repletion of albumin and  $\alpha_1$ -globulin and an initial abnormal increase in  $\beta$ - and  $\gamma$ -globulins. On the other hand, with the optimal intake of B vitamins, the fractions are restored to normal level.

3. *Influence of vitamin B<sub>12</sub> and folic acid on depletion and repletion of liver cell constituents in the protein fasted and refed rat:* The participation of vitamin B<sub>12</sub> in intermediary metabolism, though widespread and well recognized, is as yet ill-defined. Folic acid is known to be involved in several one-carbon addition and transfer reactions<sup>42</sup>. Several reports point to a close relationship of folic acid and vitamin B<sub>12</sub> between themselves and with choline and methionine<sup>43, 44</sup>. Towards better understanding of the relationship of folic acid and vitamin B<sub>12</sub> to protein metabolism, a study was undertaken on the effect



TABLE III

Rate of regeneration (after 2 weeks of protein fasting) on 10 per cent casein at 2 levels of B vitamins:  
Changes in serum proteins and their electrophoretically separated fractions

Group		Vitamin level	Total protein	Albumin	Globulin			
					$\alpha_1$	$\alpha_2$	$\beta$	$\gamma$
			Grams per 100 ml serum					
Protein-fasted (14 days)	...	Optimal	5.40 $\pm 0.34$	2.12 $\pm 0.16$	0.86 $\pm 0.04$	0.56 $\pm 0.16$	0.69 $\pm 0.03$	1.16 $\pm 0.11$
Control	...	Optimal	6.80 $\pm 0.11$	2.82 $\pm 0.02$	1.31 $\pm 0.09$	0.67 $\pm 0.05$	0.89 $\pm 0.01$	1.09 $\pm 0.02$
		Minimal	6.85 $\pm 0.15$	2.50 $\pm 0.03$	1.03 $\pm 0.01$	0.82 $\pm 0.10$	1.08 $\pm 0.02$	1.34 $\pm 0.09$
Refed protein (4 days)	...	Optimal	5.85 $\pm 0.01$	2.01 $\pm 0.03$	1.04 $\pm 0.06$	0.77 $\pm 0.07$	0.92 $\pm 0.10$	1.03 $\pm 0.11$
		Minimal	6.90 $\pm 0.05$	2.30 $\pm 0.02$	1.04 $\pm 0.02$	0.68 $\pm 0.03$	1.25 $\pm 0.02$	1.62 $\pm 0.06$
Refed protein (11 days)	...	Optimal	7.30 $\pm 0.10$	2.84 $\pm 0.12$	1.25 $\pm 0.12$	0.82 $\pm 0.11$	1.15 $\pm 0.12$	1.19 $\pm 0.05$

The animals were protein starved for 2 weeks and regeneration was studied at the end of 4 and 11 days of refeeding protein.  
For details, see footnote Tables I and II.

of these vitamins on the rate of regeneration in the rat of liver cytoplasmic constituents depleted by protein fasting and refed a 10 per cent casein diet. The animals were made deficient by feeding first iodinated casein (0.15 per cent) and then succinylsulfathiazole (2 per cent) in a 10 per cent casein diet free from these two vitamins.

The vitamins-fed animals show greater losses in weight during protein starvation (Table IV), but weight recovery in these animals during protein refeeding is faster than in the deficient ones. The increased gain in weight resulting from supplementing the casein diet with vitamin B<sub>12</sub> and folic acid may indicate a more efficient utilization of dietary constituents, as these vitamins are known to be involved in the metabolism of several amino acids, notably methionine, tryptophan and histidine and, probably, of threonine as well<sup>43</sup>. The loss of N from liver is comparable in both the deficient and the vitamins-fed animals. The non-protein nitrogen (NPN) content of the homogenate is also reduced in protein fasted animals. While nuclear and mitochondrial fractions contribute to this effect, there is an increase in NPN in the supernatant fraction. The increase in NPN of the supernatant on protein fasting would point to a breakdown of soluble proteins, among others xanthine oxidase. The higher concentration of plasma proteins in the vitamins-fed animals would suggest better utilization of proteins from the diet<sup>45</sup>. The plasma protein and NPN contents



TABLE IV

*Influence of vitamin B<sub>12</sub> and folic acid on growth and liver constituents during protein-fasting and refeeding*

Group	Animal weight g.	mg./g. fresh weight			Plasma total protein g/100 ml.	Plasma NPN mg/100 ml.	Alkaline phosphatase (nuclei) $\mu$ g/hr./g. fresh liver	$\mu$ l O <sub>2</sub> uptake/hr/g. fresh liver		Total SH mg./100g. fresh liver	Co A units/g. fresh liver	$\mu$ g. per gram fresh liver		
		Liver N Homogenate	Liver NPN					Succin oxidase (Mitochondria)	Xanthine oxidase (supernatant)			TPP	FAD	PN
			Homogenate	Super-natant										
Without vitamin B <sub>12</sub> and folic acid														
Protein-fasted (7 days) ...	103.2 $\pm$ 1.6	13.3 $\pm$ 0.22	2.05 $\pm$ 0.03	1.66 $\pm$ 0.05	3.55 $\pm$ 0.21	26.45 $\pm$ 0.85	285 $\pm$ 17	185 $\pm$ 18	nil	24.3 $\pm$ 1.9	123 $\pm$ 2	12.6 $\pm$ 1.16	10.2 $\pm$ 0.85	412 $\pm$ 12
Regenerated (7 days) ...	128.4 $\pm$ 4.0	16.1 $\pm$ 0.14	2.43 $\pm$ 0.02	1.29 $\pm$ 0.05	3.81 $\pm$ 0.34	32.27 $\pm$ 1.52	511 $\pm$ 26	345 $\pm$ 15	nil	40.2 $\pm$ 2.1	161 $\pm$ 5	15.2 $\pm$ 1.01	15.9 $\pm$ 0.75	415 $\pm$ 14
Control ...	124.5 $\pm$ 3.0	17.9 $\pm$ 0.11	2.20 $\pm$ 0.06	1.34 $\pm$ 0.06	4.04 $\pm$ 0.31	29.35 $\pm$ 1.04	559 $\pm$ 23	488 $\pm$ 39	47.2 $\pm$ 9.6	82.1 $\pm$ 1.1	209 $\pm$ 7	16.5 $\pm$ 0.82	19.5 $\pm$ 1.42	470 $\pm$ 22
With vitamin B <sub>12</sub> and folic acid														
Protein-fasted (7 days) ...	106.8 $\pm$ 2.1	14.6 $\pm$ 0.17	1.89 $\pm$ 0.01	1.46 $\pm$ 0.04	3.63 $\pm$ 0.07	20.52 $\pm$ 1.55	340 $\pm$ 19	264 $\pm$ 21	nil	40.5 $\pm$ 2.4	118 $\pm$ 3	12.8 $\pm$ 1.05	11.8 $\pm$ 1.55	468 $\pm$ 9
Regenerated (7 days) ...	142.2 $\pm$ 7.3	17.2 $\pm$ 0.25	2.16 $\pm$ 0.04	1.14 $\pm$ 0.03	4.45 $\pm$ 0.52	25.44 $\pm$ 0.92	610 $\pm$ 25	572 $\pm$ 29	97.1 $\pm$ 5.4	61.2 $\pm$ 1.2	142 $\pm$ 8	19.0 $\pm$ 0.88	26.7 $\pm$ 2.36	508 $\pm$ 12
Control ...	137.5 $\pm$ 6.1	18.6 $\pm$ 0.13	2.05 $\pm$ 0.02	1.18 $\pm$ 0.04	4.87 $\pm$ 0.14	27.15 $\pm$ 0.84	632 $\pm$ 15	670 $\pm$ 14	156.8 $\pm$ 6.6	92.3 $\pm$ 1.0	158 $\pm$ 6	19.5 $\pm$ 1.22	28.4 $\pm$ 3.15	555 $\pm$ 16

Animals weighing about 60g, were initially depleted of vitamin B<sub>12</sub> and folic acid reserves by maintenance on a purified ration consisting of (percentages) hot alcohol extracted casein 10, iodinated casein (protomone: Cerophyl Laboratories, Kansas City, MO) 0.1, sesame oil 8, sucrose 5, maize starch 72.9 and salt mixture (U.S.P. No. XIV) 4, vitamin additions to the diet were (mg. per kg. of diet): thiamine hydrochloride 6, riboflavin 10, nicotinic acid 30, calcium pantothenate 20, pyridoxine hydrochloride 6, biotin 1, choline chloride 500, inositol 500, 2-methyl 1, 4-naphthaquinone 10,  $\alpha$ -tocopherol 50 and vitamin A 5000 I.U. At the end of 2 weeks, iodinated casein was withdrawn and 2 per cent succinylsulfathiazole was incorporated in the diet at the expense of starch. Vitamins fed animals received in addition to a supplemental of vitamin B<sub>12</sub> (150 g/kg. of diet) and folic acid (5 mg./kg. of diet). Other details as under Tables I and II.



are also reduced in protein starvation. Refeeding protein, however, repletes liver N. The NPN concentration of homogenate and its nuclear and mitochondrial fractions are repleted while that of supernatant is lowered. The plasma proteins are nearly restored to levels prior to protein starvation. The value for NPN, on refeeding protein in the deficient animals is more than the control level while in the vitamins fed animals it is lower. The lowering of NPN in the vitamins-fed animals may indicate a better utilization of the free amino acids for protein synthesis. A coenzymic role for vitamin B<sub>12</sub> in protein synthesis has been claimed<sup>46-51</sup>, but does not appear to be so<sup>52, 53</sup>.

The activities of alkaline phosphatase, succinoxidase and xanthine oxidase are lowered in the deficient animals. A marked reduction in xanthine oxidase activity in vitamin B<sub>12</sub> deficiency has been shown<sup>54, 55</sup>. It has also been demonstrated that the quantities of liver total riboflavin and FAD are decreased in B<sub>12</sub> deficiency<sup>56</sup>. The enzyme activities are further reduced in protein deprivation, xanthine oxidase being reduced to zero. The lowering of xanthine oxidase in protein-fasted animals might represent a depletion of the cofactor and/or the apoenzyme protein. However, since during protein starvation, the supernatant FAD is not appreciably affected, it would seem that apoenzyme moiety mainly reflects protein nutrition. Refeeding of protein results in recovery of alkaline phosphatase and succinoxidase in both groups while xanthine oxidase is partially restored in only the vitamins-fed animals. The availability of methionine through dietary protein is known to determine to a large measure the regeneration of tissue xanthine oxidase. The partial restoration of xanthine oxidase during regeneration in vitamin B<sub>12</sub> and folic acid fed animals could thus be due, in part, to the known involvement of B<sub>12</sub> in the biosynthesis of methionine.

Higher content of soluble sulfhydryl is observed in the vitamin B<sub>12</sub> and folic acid-fed animals. In vitamin B<sub>12</sub> deficiency, low sulfhydryl values for rat livers have been reported. Repletion of SH levels is greater in the supplemented group and a close relationship between vitamin B<sub>12</sub> and sulfhydryl metabolism has been shown. Dubnoff<sup>58</sup> has suggested that vitamin B<sub>12</sub> may play a role in the reduction of S-S groups.

The vitamins-fed animals have lower liver CoA content as compared to the deficient ones. Such an inverse relationship has also been observed by Wong and Schweigert<sup>37</sup>. It has been explained as a physiological adaptive mechanism to increase energy production by providing more C<sub>2</sub> units from fatty acid oxidation and more effective concentration of the coenzyme to participate in the reactions of the Krebs cycle. The depletion of CoA in the deficient animals is of a greater order than the vitamins-fed rats. Refeeding protein increases CoA content more in the deficient animals. The lower concentration of TPP, FAD and PN in the deficient animals could result from a reduced synthesis or an increased breakdown of these nucleotides. Such a possibility has also been proposed by Dianzani<sup>15, 59</sup> with respect to changes in PN and TPP observed in fatty livers from choline deficient or carbon tetrachloride poisoned rats. Vitamin B<sub>12</sub> deficiency has been shown to result in decreased quantities of PN, FAD and TPP<sup>33, 34</sup> accompanied by a lowered rate of synthesis of these cofactors from their respective vitamins administered intraperitoneally. Protein fasting depletes the contents of these cofactors. During regeneration, greater repletion is achieved in the vitamins supplemented group. The extent of depletion of TPP from the supernatant FAD from mitochondria and PN from the supernatant would point to differences in the manner and extent to which each fraction of the cell may be affected in protein starvation.



4. *Changes in plasma nitrogen and certain liver constituents in the protein-fasted and refed rat in relation to quality of dietary protein:* The protein content of liver is to a certain extent a function of the quantity and nutritive value of dietary protein. This is best seen during replenishment of tissue proteins in the protein-fasted animals<sup>60</sup> and is a basis for quantitatively assaying protein quality<sup>61</sup>. In the present work, a comparison has been made of a wheat-Bengal gram:diet I (60:18) with one based on wheat-Bengal gram-egg albumen:diet II where egg albumen (2 per cent) replaced an equivalent amount of legume protein, at 10 per cent protein level with respect to plasma and liver nitrogen as well as certain liver enzymes and cofactors during protein deprivation and subsequent realimentation.

The data on body weights (Table V) indicate that wheat-Bengal gram-egg albumen diet supports better growth as seen from the weights of the control groups, although the weight losses during one week protein fasting and gains on refeeding protein are almost similar in both the groups. In fact, the superiority of the egg albumen supplemented diet is seen in every respect studied, viz., the levels of plasma and liver proteins, liver enzyme activities and cofactors. Kosterlitz<sup>62</sup> has shown that liver N is a function of the amount of protein fed as well as the type of protein. It may be expected, therefore, that with improvement in protein quality, a faster rate of repletion of cellular constituents will result. The nitrogen content of livers of animals on diet I is lower than that on diet II. Protein fasting results in N loss which is contributed by all fractions. Refeeding does not restore this loss fully during 7 days. This is also borne out by the plasma protein content. That egg albumen is superior to casein, so also casein to peanut protein, has been demonstrated from their effects on serum proteins<sup>63</sup>. The concentration of plasma proteins is somewhat lower in the animals fed diet I, whether protein depleted, refed or control, as compared to the corresponding groups receiving diet II.

The activities of alkaline phosphatase, succinoxidase and xanthine oxidase and the SH contents are seen to be higher in animals fed diet II. Alkaline phosphatase and succinoxidase are not affected to the same extent as xanthine oxidase by dietary inadequacies. The labile nature of xanthine oxidase has enabled its use in rating dietary protein quality<sup>64,65</sup>. Also xanthine oxidase activity has been shown to parallel the changes in liver N. Studies with amino acid diets<sup>66</sup> have shown that xanthine oxidase activity is influenced by the deficiency of amino acids in the diet. The low xanthine oxidase activity obtained with diet I is also indicative of its poor quality as compared to diet II. It is possible that dietary inadequacies are better reflected in the soluble proteins. Appreciable changes in tissue sulfhydryl content occur in association with alterations in the dietary content of sulfur amino acid<sup>67</sup>. The higher concentration of the SH with the egg albumen supplemented diet could be the result of an increased supply of sulfur amino acids in the diet.

The contents of TPP, FAD, PN and CoA are also influenced by protein quality. Increased concentration of these cofactors with egg albumen supplements to wheat-Bengal gram diet are observed. In protein starvation, the contents of TPP, FAD, PN and CoA are reduced and diet II brings about better restoration of these than diet I. It would seem, therefore, that the contents of cofactors in the liver are determined by the quality of the dietary protein. It has also been observed that the low liver PN content in protein deficiency<sup>68</sup> is due to a reduced capacity of the liver to synthesize it under conditions of protein deficiency.

5. *Relationship of protein quality and quantity on the repletion of certain liver constituents in the protein-fasted rat:* Earlier studies have indicated that protein starvation



TABLE V  
Influence of protein quality on body weight and liver constituents during protein fasting and refeeding

Group	Animal weight (g)	Liver N mg./g. fresh weight	Plasma proteins mg./100 ml.	Liver SH mg./100g. fresh weight	Alkaline phosphatase (nuclei)/ $\mu$ g. liberated/g. fresh liver	$\mu$ l O <sub>2</sub> uptake/hr./g. fresh liver		$\mu$ g. per gram fresh liver			CoA units per g. fresh liver
						Succinoxidase (mitochondria)	Xanthine oxidase (supernatant)	TPP	FAD	PN	

Wheat-Bengal gram diet (Diet I)											
Protein fasted (7 days) ...	115.0 $\pm$ 1.6	19.1 $\pm$ 0.34	5.02 $\pm$ 0.21	25.8 $\pm$ 1.35	242 $\pm$ 24	357 $\pm$ 18	nil	10.5 $\pm$ 1.64	8.9 $\pm$ 0.24	807 $\pm$ 14	101 $\pm$ 4
Refed (7 days) ...	132.0 $\pm$ 3.2	21.4 $\pm$ 0.24	6.40 $\pm$ 0.23	98.2 $\pm$ 3.85	482 $\pm$ 17	592 $\pm$ 32	130 $\pm$ 8	13.6 $\pm$ 0.92	12.2 $\pm$ 0.86	964 $\pm$ 25	145 $\pm$ 3
Control ...	126.0 $\pm$ 2.8	24.8 $\pm$ 0.32	6.81 $\pm$ 0.31	148.0 $\pm$ 7.18	486 $\pm$ 25	684 $\pm$ 26	185 $\pm$ 21	14.2 $\pm$ 1.76	18.2 $\pm$ 1.35	1034 $\pm$ 36	155 $\pm$ 7

Wheat-Bengal gram-egg albumen diet (Diet II)											
Protein fasted (7 days) ...	125.0 $\pm$ 1.9	22.8 $\pm$ 0.73	5.60 $\pm$ 0.03	44.7 $\pm$ 0.46	295 $\pm$ 7	406 $\pm$ 31	nil	11.9 $\pm$ 0.88	9.8 $\pm$ 0.38	842 $\pm$ 19	113 $\pm$ 2
Refed (7 days) ...	146.0 $\pm$ 4.5	25.0 $\pm$ 0.56	6.94 $\pm$ 0.13	126.4 $\pm$ 8.15	533 $\pm$ 15	656 $\pm$ 25	172 $\pm$ 15	15.2 $\pm$ 0.65	14.6 $\pm$ 0.93	1034 $\pm$ 26	162 $\pm$ 4
Control ...	138.0 $\pm$ 4.2	27.6 $\pm$ 0.84	7.62 $\pm$ 0.24	176.6 $\pm$ 11.38	521 $\pm$ 26	745 $\pm$ 18	265 $\pm$ 27	16.1 $\pm$ 1.56	22.3 $\pm$ 1.85	1149 $\pm$ 32	178 $\pm$ 6

Diet I contained (in percentages): wheat flour 60, Bengal gram (*cicer arietinum*) flour 18, salt mixture (U.S.P. No. XIV) 4, sesame oil 8, vitaminised sucrose 1 and starch 200. Diet II was the same as diet I modified by addition of 2 per cent egg albumen which replaced an equivalent amount of the legume protein, the difference being made up with starch. The vitamins added to both the diets were (mg. per kg. of diet): thiamine hydrochloride 6, riboflavin 10, nicotinic acid 30, calcium pantothenate 20, pyridoxine hydrochloride 6, p-aminobenzoic acid 100, biotin 1, inositol 500, folic acid 5, vitamin B<sub>12</sub> 0.2, choline chloride 500,  $\alpha$ -tocopherol 50, vitamin K 10, and vitamin A 5000 I.U.; other details as under Tables I and II.



causes depletion of nitrogenous constituents, certain enzymes and cofactors of liver. A study was undertaken to investigate the relationship of protein quality and quantity on the enzyme activity, the cofactor concentration and the vitamin content in addition to other liver cell constituents during realimentation of 7 days using casein at 10 or 18 per cent and  $\alpha$ -protein also at 18 per cent after 7 days of protein fasting.

Maximum gains in body weight (Table VI) are seen with 18 per cent casein diet, while the increases with 10 per cent casein diet and 18 per cent  $\alpha$ -protein diet are almost comparable. It is seen that feeding the vegetable protein at 18 per cent compensates for its poor quality. These changes in body weight are also reflected on certain liver cell constituents. Liver N is completely repleted in groups fed 18 per cent casein or  $\alpha$ -protein. The liver N values would point to the superiority of casein over  $\alpha$ -protein at the same protein level, but  $\alpha$ -protein at 18 per cent would compare favourably with casein at 10 per cent.

Changes in the activities of succinic dehydrogenase, xanthine dehydrogenase and malic dehydrogenase parallel the total protein changes. Again, the enzyme activities are repleted better with casein than with  $\alpha$ -protein at 18 per cent. Observations on xanthine dehydrogenase indicate that it is not as completely removed as xanthine oxidase during protein fasting. Similar observations with reference to xanthine oxidase and xanthine dehydrogenase have been made by others<sup>69, 70</sup>. The ability to synthesize citrovorum factor from added folic acid is appreciably impaired in protein starvation. Refeeding protein for one week far from completely restores this. In a recent study of folic acid metabolism, Guggenheim and Halevy<sup>71</sup> have shown that animals maintained on protein free diets or diets containing nutritionally inferior proteins have lower concentrations of liver folic acid and citrovorum factor.

The influence of protein quality is also reflected on the content of liver cofactors. During refeeding of protein, the depleted levels of TPP, FAD, PN and CoA are nearly restored except that of CF even with 10 per cent casein diet. With 18 per cent protein diets, restoration is more complete, casein being superior to  $\alpha$ -protein.

The changes in the vitamin contents studied parallel those in cofactors. The values for thiamine, riboflavin, nicotinic acid, pantothenic acid and folic acid are decreased during protein deprivation and are replenished on refeeding of protein, restoration being more at the 18 per cent level. A relationship has been shown to exist between protein intake and liver concentrations of certain B vitamins. Liver riboflavin has been shown to be decreased in rats maintained on low protein diets<sup>72, 73</sup> or on protein fasting. It has also been observed that liver nicotinic acid content is dependent on tissue protein<sup>32</sup>. These observations would suggest that the vitamins may exist in the liver as protein complexes.

## Summary

Protein starvation causes a loss of liver N which is contributed by all the cell fractions, inclusive of nuclear, as seen from the decrease in arginine: DNA ratio. Concomittant with the loss in N, reductions in liver SH, certain enzyme activities and cofactors are also observed. Tissue vitamin levels are also decreased. Plasma protein concentration and its electrophoretically separated fractions are lowered in protein deprivation. The effects of protein fasting for a period of 2 weeks are more severe than for one week and hence a partial repletion of tissue constituents is seen on feeding protein.



TABLE VI  
Effect of refeeding casein or  $\alpha$ -protein diets to protein fasted rats on body weights and liver constituents

Group	Animal weight (g)	Liver N mg/g fresh weight	$\mu$ l. O <sub>2</sub> uptake/hr/g fresh liver			$\mu$ g. per gram fresh liver					CoA units per g fresh liver	$\mu$ g. per gram fresh liver				
			Succinic dehydro- genase	Xanthine dehydro- genase	Malic dehydro- genase	Wet CF conver- sion	TPP	FAD	CF	Total PN		Thiamine	Riboflavin	Nicotinic acid	Pantothe- nic acid	Folic acid
Protein-fasted (7 days)	97.0 ± 2.1	20.3 ± 0.11	3560 ± 132	47 ± 13	1495 ± 265	0.45 ± 0.02	13.1 ± 1.11	9.8 ± 0.56	0.26 ± 0.03	699 ± 27	109 ± 5	15.3 ± 1.22	17.2 ± 1.62	81 ± 5	79 ± 11	0.66 ± 0.03
Regenerated (7 days) 10% casein	107.0 ± 1.8	22.8 ± 0.35	6314 ± 236	230 ± 55	2580 ± 184	0.83 ± 0.05	19.6 ± 1.81	17.2 ± 1.18	0.85 ± 0.04	1066 ± 37	155 ± 7	21.8 ± 2.54	24.7 ± 1.45	206 ± 18	126 ± 18	1.25 ± 0.03
Regenerated (7 days) 18% casein	118.0 ± 2.2	22.1 ± 0.78	8330 ± 294	367 ± 32	2872 ± 310	0.90 ± 0.06	28.1 ± 1.33	23.7 ± 1.56	0.93 ± 0.06	1159 ± 31	188 ± 6	24.6 ± 1.86	29.7 ± 2.52	257 ± 17	150 ± 9	1.36 ± 0.05
Regenerated (7 days) 18% $\alpha$ -protein	108.0 ± 1.3	26.6 ± 2.24	7456 ± 246	275 ± 64	2535 ± 392	0.75 ± 0.05	19.9 ± 1.17	20.9 ± 1.42	0.84 ± 0.05	1002 ± 18	140 ± 11	22.4 ± 1.82	26.7 ± 2.23	192 ± 15	119 ± 6	1.16 ± 0.08
Control 10% casein	113.0 ± 2.5	26.0 ± 0.28	7484 ± 281	418 ± 47	2832 ± 283	1.46 ± 0.09	20.1 ± 0.95	19.1 ± 0.95	1.35 ± 0.11	1047 ± 14	171 ± 12	22.4 ± 1.55	25.3 ± 1.64	209 ± 13	146 ± 7	1.72 ± 0.16

The 10 per cent casein diet consisted of (percentages): hot alcohol extracted casein 10, starch 70, cellulose 3, salt mixture (U.S.P. No. XIV) 4, sesame oil 8 and vitaminised sucrose 5. Vitamin addition to the diet were (mg/kg. of diet): thiamine, hydrochloride 6, riboflavin 10, nicotinic acid 30, calcium, pantothenate 20, pyridoxine hydrochloride 6, p-aminobenzoic acid 100, biotin 1, inositol 500, folic acid 5, vitamin B<sub>12</sub> 0.2, choline chloride 500,  $\alpha$ -tocopherol vitamin K 10 and vitamin A 5000 I.U. In the 18 per cent casein diet, casein was added at the expense of starch. 18 per cent  $\alpha$ -protein diet was prepared by replacing casein in the above diet.

Other details as under Tables I and II.



Higher intakes of B vitamins during refeeding of protein result in an enhanced regeneration of the tissue. The beneficial effects specifically of folic acid and vitamin B<sub>12</sub> during, protein fasting and refeeding of protein are also seen.

The superiority of wheat-Bengal gram-egg albumen over the vegetable protein diet (wheat-Bengal gram) and of casein over  $\alpha$ -protein for the repletion of cellular constituents is observed.

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# SUPRARENAL CYTOPLASMIC PROTEIN PATTERN IN NORMAL AND IN COLD STRESSED RATS

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In cold exposure, changes such as, an increase in the phosphorous containing organic compounds<sup>1</sup>, riboneucleic acid<sup>1, 2</sup> and decrease in cholesterol<sup>3, 4</sup> ascorbic acid<sup>5, 6</sup>, glycogen<sup>7</sup> and sudanophilic substances<sup>8</sup>, of the adrenal glands of rats have been reported. Cortical hypertrophy<sup>9, 10</sup> and increase in weight of the adrenal glands<sup>11, 12</sup> have also been observed in rats placed in cold temperature. Assuming that protein studies might reveal some aspect of the functional status of the cold-activated gland, investigations were undertaken and the study of adrenal cytoplasmic proteins is reported here.

## Experimental Methods and Materials

Both male and female albino rats weighing from 56-166 gms and maintained in the laboratory condition for at least 10 days on a stock diet supplemented with vitamins were taken for the experiment and divided into three groups. The 0 group rats which were kept at ordinary laboratory temperature (27-29°C) served as the normal, and the rest, groups A and B, were placed in a cold environment (6-7°C) for 24 and 48 hours respectively. After the termination of the experimental periods, rats were killed by decapitation and adrenal glands were removed quickly, chilled, pooled and weighed in a torsion balance and homogenised for 5 minutes in ice-cold phosphate buffer (0.023 M) at pH 7.4 with 0.145 M sucrose<sup>13</sup> in the proportion of 1:9. The time needed for homogenising was determined by a preliminary experiment with microscopic checking. The small amount of available homogenates was taken in special capillary tubes of about 1mm bore, and cold-centrifuged for 15 minutes at 3000 r.p.m. The clear fluid below the lipid layer of the centrifuged homogenate was carefully taken for the study. In the paper electrophoretic study Whatman No. 1 filter paper, barbiturate buffer of pH 8.6 and ionic strength 0.03 were used and a 4-hour run was allowed at 8 mA current per 16 cm breadth of the paper. Proteinograms were stained by bromophenol blue method, and subsequent scanning was carried out in a densitometer. Areas of the proteinograms were measured by a planimeter. Proteins were estimated by the standard micro-Kjeldahl method.

## Results and Discussion

The proteinograms of adrenal cytoplasm consist of three distinct characteristic zones 'X', 'Y' and 'Z' and marked variations in these zones have been observed in cases of 0, A and B groups. In every case, zone 'X' has a sharp peak, descending to zone 'Y' which is almost flat, while zone 'Z' appears as a broad big hump.

Height of zone 'X' is maximum in the A series and in the B series it is comparatively low and tends to split up into two subzones 'X<sub>1</sub>' and 'X<sub>2</sub>'. Zone 'Y' of the B series is narrower than in the other series, while zone 'Z' is similar in the three series.



# PROTEINOGRAM OF ADRENAL CYTOPLASM

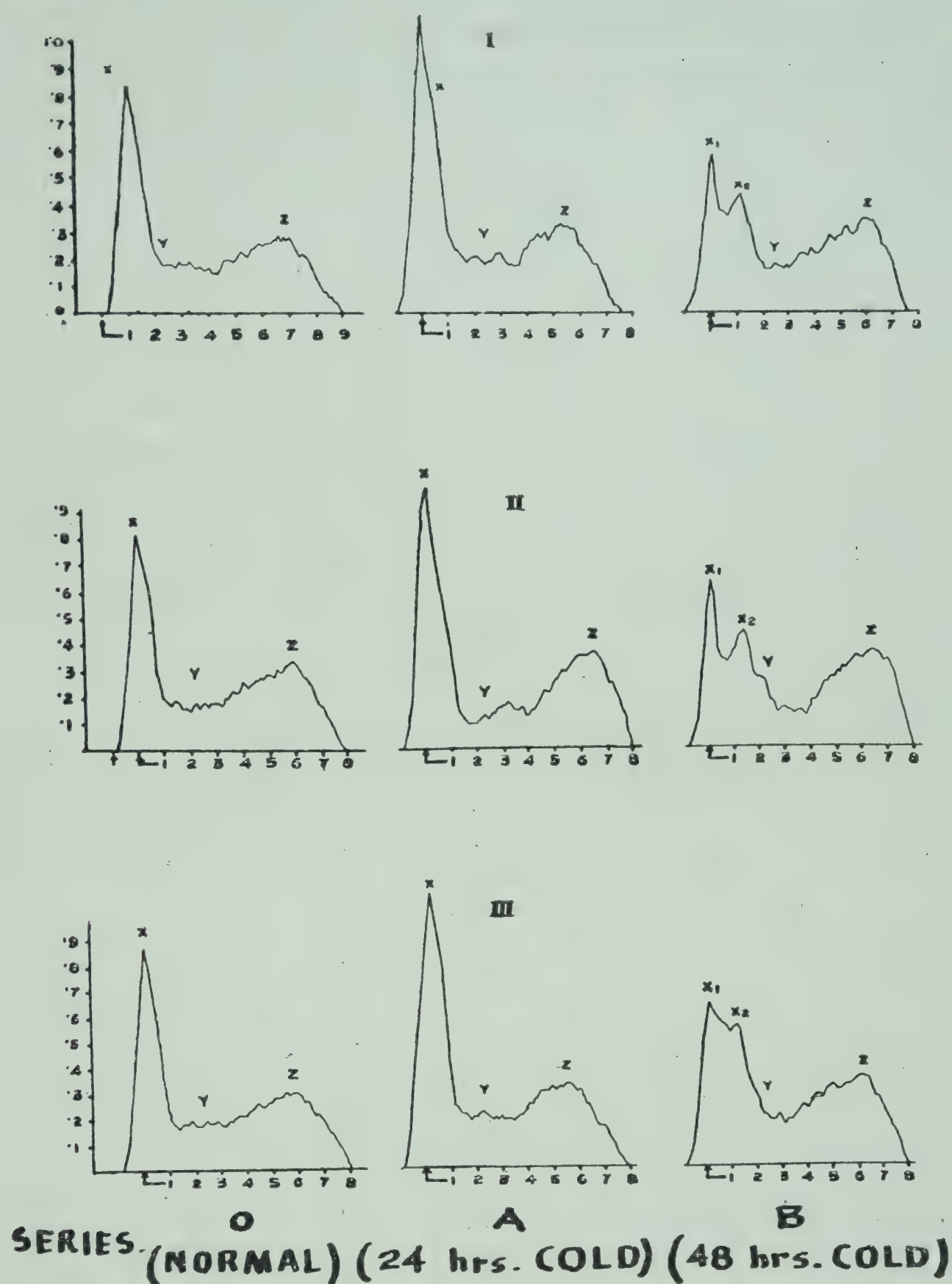


FIG. 1

The planimetric measurements show that the area of zone 'X' in the B series is smaller than that of A series but always greater than that of O series. It is also found that the area of 'X' in the A series is the greatest of all the areas measured.

The area of 'Y' is the smallest in the B series, but no characteristic change in its areas has been observed in the O and A series. Regarding the area of zone 'Z' only a small increase in the B series is noticed.

A maximum increase in the total area is noticed in the proteinograms of A group and though the total area of the B group is less than that of the A group, it is greater than that of the O group.



TABLE I

Measurement of area of adrenal cytoplasmic proteinograms of Fig. 1

No. of expt.	Cold exposure (hr.)	Total area (sq. cm.)	Fraction 'X' (sq. cm.)	Fraction 'Y' (sq. cm.)	Fraction 'Z' (sq. cm.)
I	0	22.3	9.1	3.6	9.6
	24	26.2	13.1	3.7	9.4
	48	22.5	9.7	1.3	11.5
II	0	23.0	8.5	3.3	11.2
	24	26.0	12.3	3.0	10.7
	48	24.6	11.5	1.7	11.4
III	0	23.3	9.1	3.3	10.9
	24	28.1	14.3	3.4	10.4
	48	27.0	12.7	2.3	12.0

TABLE II

Cytoplasmic and total protein content in adrenal gland (%) from normal and cold stressed rats

No. of expt.	Cold stress (hr.)	Total protein in homogenate (g %)	Cytoprotein in homogenate (g %)	Calculated nuclear protein in homogenate (g %)	Total protein cytoplasmic protein
I	0	2.16	1.05	1.11	2.05
	24	2.2	1.26	0.96	1.75
	48	1.92	1.10	0.82	1.75
II	0	2.28	1.10	1.18	2.07
	24	2.33	1.34	0.99	1.74
	48	2.27	1.19	1.08	1.90
III	0	2.20	1.02	1.18	2.15
	24	2.52	1.41	1.11	1.78
	48	2.32	1.21	1.11	1.91

From the data obtained by chemical analysis it is found that maximum increase in the total adrenal cytoplasmic protein per gram of tissue occurs after 24 hours cold exposure. The cytoplasmic protein value after 48 hours cold exposure is higher than the normal value but always lower than that of A series. The total protein values per gram of tissue of the 48 hours-cold-exposed rats are variable. The calculated protein value of the nucleus shows that the percentage of protein per gram of nucleus in the normal glands is higher than in the other two groups.

Calculation of total: Cytoplasmic protein values also clearly indicate that in cold exposed rats the relative proportion of total protein to cytoplasmic protein is less than that in the normal rats.



From the results in this experiment it is concluded that the metabolically active glands of cold-exposed rats are intimately associated with protein synthesis inside the gland; increase of protein content is noted in the cytoplasm only and not in the nucleus of the adrenal cells. Moreover different proteins of the cytoplasm do not increase to the same extent as shown by the fact that three fractions of cytoplasmic protein separated by paper electrophoresis do not increase equally in cold stress. Most marked increase is noted in fraction 'X'. Rise of a second peak in fraction 'X' in 48 hour cold-stressed group suggests that this fraction undergoes not only a quantitative change in cold-stress but its character also is affected. Further study is needed to elucidate the nature of the proteins of the different fractions separated in adrenal cytoplasm from normal and cold stressed rats.

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# COMPARATIVE TRANSAMINATION CAPACITY ON COLD EXPOSURE

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Animals are now divided into three groups according to their power to regulate their body temperature in the face of a change in the ambient temperature. Most of the mammals are homeothermic and they can maintain a constant body temperature. Most of the invertebrates and the non-mammal invertebrates are poikilothermic—their body temperature is not constant, but changes with that of the ambient temperature and the intermediate stenothermic birds and baby mammals can regulate body temperature only when the changes in ambient temperature are small<sup>1</sup>. These three groups of animals behave differently in the pattern of their tissue respiration and the evolution of thermoregulation in these species is reflected in the changes of respiratory enzymes<sup>2</sup>. In homeotherms acetate oxidation has evolved more in the liver and kidney compared to that of succinate oxidation. When exposed to low ambient temperature, there is a depression of respiration which is located mostly in the flavoprotein or preflavoprotein systems, while there is an augmentation of respiration by the cytochrome systems in poikilothermic toads and stenothermic birds under cold exposure. With this difference in oxidation pattern, it was thought worthwhile to study the amino acid oxidation and transamination reactions in these species of animals on cold exposure.

## Experimental

For homeothermic group, albino rats of either sex of 150-200 gm. body weight, for the stenothermic group pigeons of 200-250 g. weight and for the poikilothermic group, toads, were used. Thirty-two animals of each species were divided into two groups, one group was kept in cages at 25°C ambient temperature (Control group) and the others at 0°C in a refrigerator for 2-1/2 hours (Cold group). After this exposure the animals were sacrificed, their liver and kidney were homogenized at 10 per cent level with cold isotonic KCl solution. 0.4 ml of homogenate was used in Warburg flasks with 0.5 ml of 0.5 M. sodium aspartate and 0.2 ml. of 0.5 M. sodium  $\alpha$ -ketoglutarate for the study of oxygen consumption with amino acid substrate (II) and transamination reactions (III). The gas phase used was air and after an equilibrium period of 10 minutes, oxygen measurement was made for 30 minutes and transamination reaction was studied quantitatively by measurement of oxalacetate after decarboxylation to pyruvate. Pyruvate substrate was used for Krebs cycle oxidation (I)<sup>4</sup>.

## Results and Discussion

The results of the Krebs-cycle oxidation with pyruvate substrate, oxygen consumption with 0.2 M. sodium aspartate substrate and the transamination capacity expressed as  $\mu$ g pyruvate formed by decarboxylation of oxaloacetate after 30 minutes reaction and



TABLE I

The  $QO_2 N$  (oxygen consumption in  $\mu l$  per mg N per hour) of liver and kidney homogenates of rats, pigeons and toads at 25°C (control) and those exposed to 0°C for 2-1/2 hours (cold) with Krebs cycle oxidation pyruvate substrate (I), with 0.2 M sodium aspartate substrate (II) and transamination capacity expressed as  $\mu g$ . pyruvate formed by decarboxylation of oxaloacetate<sup>3</sup> after 30 minutes' reaction per hour per mg tissue nitrogen (III).  $\mu$  value is calculated on the basis of ambient temperature difference of 0-25°C. Average of 16 animals in each group.

TISSUE		LIVER				KIDNEY			
Subject	Reaction	Control	Cold	% Change	$\mu$ Value	Control	Cold	% Change	$\mu$ Value
Rat	I	24.6 $\pm$ 2.8	20.9 $\pm$ 1.8	- 15.1	32 $\pm$ 7	36.0 $\pm$ 2.4	29.3 $\pm$ 1.7	- 18.6	180 $\pm$ 13
	II	27.4 $\pm$ 2.5	45.9 $\pm$ 2.1	+ 67.6	268 $\pm$ 15	44.7 $\pm$ 2.9	74.0 $\pm$ 2.5	+ 65.5	259 $\pm$ 12
	III	377.1 $\pm$ 3.6	578.4 $\pm$ 3.7	+ 53.4	221 $\pm$ 5	387.8 $\pm$ 3.1	501.0 $\pm$ 3.1	+ 29.2	132 $\pm$ 6
Toad	I	29.4 $\pm$ 2.6	32.7 $\pm$ 1.2	+ 11.3	58 $\pm$ 12	57.7 $\pm$ 2.8	64.1 $\pm$ 2.3	+ 10.9	54 $\pm$ 10
	II	33.1 $\pm$ 2.4	59.5 $\pm$ 2.6	+ 79.7	303 $\pm$ 12	117.4 $\pm$ 4.3	59.7 $\pm$ 3.2	- 49.1	347 $\pm$ 14
	III	415.6 $\pm$ 5.6	557.9 $\pm$ 8.4	+ 34.3	151 $\pm$ 7	510.4 $\pm$ 5.4	668.9 $\pm$ 2.1	+ 31.1	140 $\pm$ 2.5
Pigeon	I	89.0 $\pm$ 2.4	124.3 $\pm$ 2.9	+ 39.6	172 $\pm$ 13	82.3 $\pm$ 2.3	117.9 $\pm$ 3.4	+ 43.3	186 $\pm$ 13
	II	62.4 $\pm$ 2.6	76.0 $\pm$ 2.7	+ 21.7	102 $\pm$ 12	82.7 $\pm$ 1.8	66.1 $\pm$ 2.3	- 20.1	116 $\pm$ 12
	III	183.7 $\pm$ 2.9	205.5 $\pm$ 4.2	+ 11.9	58 $\pm$ 6	231.3 $\pm$ 1.7	269.5 $\pm$ 3.6	+ 16.5	79 $\pm$ 6

expressed as the value per hour per mg. tissue nitrogen are presented in Table I. On cold exposure the Krebs cycle oxidation with pyruvate substrate is depressed by 15-19 per cent in the liver and kidney of the homeothermic rat. Previous work with succinate and ascorbate has shown that the cold depression of respiration is greater in flavoprotein or preflavoprotein systems and the present work shows the depression to be in the preflavoprotein system. A slight augmentation of Krebs cycle oxidation with pyruvate substrate of 11 per cent in toad and 40 per cent in bird tissues occurs on exposure of these poikilotherms and stenotherms to cold. This confirms the earlier work with succinate and ascorbate that the augmentation is located in the flavoprotein and cytochrome systems. Thus it is evident that in the phylogenetic evolution of thermoregulation flavoprotein and cytochrome systems were evolved earlier in the poikilotherms and stenothermic birds and the preflavoprotein systems were evolved much later in the homeothermic rat.

The oxygen consumption with amino acid substrate under cold exposure increases in rat tissues and only in the livers of birds and toad, but there is a marked depression in  $QO_2$  values in the kidneys of birds and toads. Although oxidation of amino acids is different in liver and kidney tissues, transamination capacity is increased in both liver and kidney in all the three species examined. It has not been possible to eliminate the effect of thyroid secretion which is increased in cold exposure and also the role of pyridoxin on the transamination capacity in the cold has not been studied<sup>5</sup>. From the above work, it appears that transamination reactions have evolved quite early in the phylogenetic scale, while the amino acid oxidation in the kidney has evolved only to a small extent in the homeothermic rats.



## Summary

Krebs cycle oxidation is depressed in the rat and augmented in birds and toads on cold exposure. Oxygen consumption with amino acid substrate under cold stress increases in rat tissues and only in the livers of birds and toads and depressed in the kidney of the latter two species. Transamination reaction is increased in both liver and kidney of all species on cold exposure.

## Acknowledgment

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# INTERRELATIONSHIP BETWEEN ADRENOCORTICOTROPHIC AND MELANOCYTE STIMULATING HORMONES

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The lively controversy over the interrelationship of adrenocorticotrophin (ACTH) to melanocyte stimulating hormone (MSH) was started by Sulman<sup>1</sup>, who noticed that ACTH in a very minute dose could blacken the tree frog (*Hyla arborea*). It was established at that time that among the pituitary hormones, only MSH, which had variously been designated by the term melanophore hormone, or intermedin, could provoke such a response in the amphibian. Since the dose Sulman employed for his work was infinitesimally small, he could not conceive of the response arising out of an MSH impurity associated with his ACTH preparation. He, therefore, argued that ACTH and MSH were identical. Sulman's work was strengthened by Johnsson and Hogberg<sup>2</sup>, who independently arrived at the same conclusion and cited various physico-chemical data of ACTH and MSH to prove their identity.

The contention of the above workers was soon challenged. Morris<sup>3</sup> showed that MSH was potentiated by alkali, which inactivated ACTH. He further pointed out that a partial separation of the two activities was possible. Other workers also criticised Sulman on the same points<sup>4-6</sup>.

The present authors criticised Sulman on physiological grounds. Working with mice, they found that MSH had no adrenocorticotrophic property; for, it did neither increase the adrenal weight nor could elicit any histological or histochemical response from the adrenal gland. It was further shown that MSH failed to arrest the diminishing adrenal weight of the rat following hypophysectomy<sup>8</sup>. When rats were subjected to prolonged treatment with MSH, no change in the weight or histology of the adrenal was observed<sup>9</sup>. Similar results with hypophysectomized rats and frogs were independently recorded by other workers<sup>10,11</sup> and were adduced to negate Sulman's idea.

Yet, some evidences remained which indicated that the relation between the two hormones might be of a special nature. Thus, Karkun *et al*<sup>8</sup>, observed that preparation of MSH obtained according to Landgrebe *et al*<sup>12</sup> depleted adrenal ascorbic acid in hypophysectomized rats. Since the preparation was not electrophoretically homogenous, they thought that this depletion might be due to ACTH contamination, in as much as only 0.2  $\mu$ g. of ACTH could evoke such a response. However, the time response curve of such depletion seemed not to simulate that obtained with ACTH (Acthar gel Armour) even though both the active substances were given intravenously (Karkun and Sur, unpublished data). The result appeared a bit intriguing as it was previously observed that ACTH, whether pure or impure, showed a similar time response curve in the hypophysectomized rat so long the active principles were administered intravenously into such animal. Thing<sup>14</sup> found that it both the adrenocorticotrophic and melanophorotrophic activities of a large number of ACTH preparations were assayed side by side with hypophysectomized rats and frogs



(*Xenopus laevis*) respectively, the ratio of the two activities of each preparation was always the same viz., 1.0. A similar correlation of the two activities was also reported previously<sup>10</sup>. Thing also noticed that the more highly purified adrenocorticotrophic hormone had the highest melanophorotrophic activity<sup>14</sup>. In an effort to separate the two activities by applying an electric field, Thing<sup>15</sup> subjected three different ACTH preparations to paper electrophoresis and succeeded in dissociating the activities only in 2 cases. Johnsson and Hogberg<sup>16</sup> found that in Addison's disease not only the blood titre of ACTH is elevated but also that of MSH. Administering ACTH (Corticotrophin, Armour) for prolonged periods into cats, with distinct pars intermedia, Karkun *et al*<sup>17</sup>, observed that the pars intermedia cells became hypertrophied with other changes, which indicated a stimulation of this part of the gland. A concomitant change in phosphatase activity of those cells was also observed<sup>18</sup>. Since phosphatase change is associated with protein synthesis<sup>19</sup>, it was argued that prolonged exogenous ACTH did stimulate the MSH elaborating cells thus resulting in excess secretion of the latter hormone. It was presumed that in Addison's disease, a similar state of affairs probably occurs, the ACTH effective in such cases being endogenous in nature and having its origin in the pituitary as a result of hypofunction of the adrenal<sup>20</sup>. The result with the rat seems to be a bit ambiguous. Forgacs<sup>21</sup> observed that in unilaterally adrenalectomized rats given massive doses of cortisone, the ACTH content of the pituitary was reduced to 1/10 of its previous value, but MSH content remained unchanged. In rats subjected to bilateral adrenalectomy or given ACTH treatment, the pituitary concentration of MSH remained unchanged even after 7, 14 or 21 days<sup>21a</sup>. Whether the above discrepancy could be ascribed to difference in species is yet to be seen. Interestingly enough Johnsson and Hogberg<sup>16</sup> observed an elevation of blood MSH titre in adrenalectomized rats. Elevation of ACTH titre in such animals was also recorded<sup>16</sup>.

While the physiological properties of the two hormones were thus being actively studied and compared, several groups of investigators were trying to purify the MSH extracts with a view to obtain a homogenous MSH. The first group to succeed in this line was Lerner and his collaborators who claimed to have isolated a homogenous MSH preparation from pig pituitary<sup>22</sup>. This was followed by claims advanced by Benfey and Purvis<sup>23</sup>, Porath *et al*<sup>24</sup>, and Geschwind *et al*<sup>25</sup>. Except the Porath group, who worked with an ox preparation partly purified by Landgrebe and Mitchell<sup>26</sup>, all other workers employed hog posterior pituitary powder as the starting material. In view of the fact that the assay methods employed by the different groups were different and the units were arbitrarily fixed, the potencies of the preparations are not strictly comparable. However, one interesting fact emerged out of their studies. It was found that all the above workers except Lerner and his group obtained only one MSH component whose iso-electric point was 5.2—5.8. Lee and Lerner<sup>27</sup>, on the other hand, obtained two MSH components one of which was more basic having an isoelectric point of about 10.5—11.0. It was termed  $\alpha$ -MSH. The other component simulated the preparation obtained by other workers and was termed  $\beta$ -MSH. Lee & Lerner<sup>27</sup> and Lee<sup>28</sup> tried to account for the inability of other workers in obtaining  $\alpha$ -MSH, but for sometime a controversy went on, some doubting whether  $\alpha$ -MSH is a real hormone or an artifact. Recent work, however, tends to support the existence of  $\alpha$ -MSH. Geschwind is reported to have confirmed the existence of  $\alpha$ -MSH in pig pituitary preparation. Employing paper electrophoresis under high voltage, Karkun and Landgrebe (unpublished data) were able to show the presence of both  $\alpha$  and  $\beta$ -MSH



in ox posterior pituitary preparation. Recently, Lerner and his group are reported to have isolated both the hormones from the monkey pituitary (personal communication to Dr Harris). It is interesting to note, in this connection, that all these hormones were purified by methods more or less similar to those adopted for the purification of ACTH. It was further observed that all these hormones exhibit log-dose-response lines which, when worked out from the same animal viz., *xenopus laevis*, are parallel<sup>29</sup>. An interesting property of  $\alpha$ -MSH was noticed which might help to differentiate it physiologically from  $\beta$ -MSH. (Karkun and Landgrebe—unpublished data). The time-response curve of the two hormones as evaluated on toads (*xenopus laevis*) were different,  $\alpha$ -MSH being inactivated at a greater rate than  $\beta$ -MSH. The implication of such a finding on the structural relationship of the two hormones will be apparent later on.

Both these MSH components are claimed to have no ACTH activity, but the data in support of such a claim are meagre. Interesting results were, however, obtained by those who approached the problem from the ACTH side. Winter *et al*<sup>30</sup> isolated from the protein digest of swine corticotrophin a product (corticotrophin B) which behaved as a pure substance in counter-current distribution studies. Biological assay revealed in it both the corticotrophic and melanophorotrophic potencies. Bell<sup>31</sup> obtained several corticotrophins by countercurrent distribution studies of a hog preparation and was surprised to find the persistence of nearly constant intermedin to corticotrophin ratio in all of them. A similar observation was made by Dixon<sup>33</sup>. The same investigator<sup>32</sup> inactivated corticotrophin A<sub>1</sub> by periodate treatment and reactivated the same by incubation with thioglycolic acid. MSH assay of the samples revealed that the latter activity was also inactivated and reactivated in the same manner.

All this work indicated that MSH activity observed in corticotrophin is an intrinsic property of the latter and is not due to contamination with MSH.<sup>31-33</sup>

Soon an answer to the above phenomenon was traced out. Geschwind *et al*<sup>34</sup>, subjected their  $\beta$ -MSH to chemical and enzymatic degradation and evaluated the complete structure of  $\beta$ -MSH. The same investigators previously established that the sequence Met. Glu. His. Phe. Arg. Try. Gly. existed in corticotrophin. It was noticed that  $\beta$ -MSH contained a similar heptapeptide unit with other peptide linkages on both sides of the molecule. The work of Li and his group created intense interest as it furnished an explanation for the intrinsic melanophorotrophic property of corticotrophin. Soon the structures of  $\beta$ -MSH (ox)<sup>35</sup>  $\beta$ -MSH (pig)<sup>36</sup>,  $\alpha$ -MSH (pig), and  $\beta$ -MSH (human) were worked out.

Fig. 1 (given on the nextpage) shows the structures of different melanocyte stimulating hormones along with that of corticotrophin. A glance at these structures will reveal the following interesting points:

- (i) The heptapeptide Met . . . Gly. is common to all the molecules, including that of corticotrophin.
- (ii)  $\alpha$ -MSH (pig) reveals a closer chemical structural relationship to corticotrophin in as much as such similarity extends to a sequence of 13 amino acids.
- (iii) With  $\beta$ -MSH (pig) the similarity with corticotrophin extends only to a sequence of 7 amino acid residues and, but for the transposition of lysine and serine residues between the position 6 and 14 in  $\beta$ -MSH, the common sequence would have extended to 11 amino acid residues.



FIGURE 1

*Amino acid sequence of  $\alpha$ - and  $\beta$ -MSH (from the different pituitaries) compared with the amino acid sequence of corticotrophin*

Corticotrophin (pig)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	39
	H-Ser	Tyr	Ser	Met	Glu	His	Phe	Arg	Try	Gly	Lys	Pro	Val	Gly	Phe-OH
$\alpha$ -MSH (pig)	1	2	3	4	5	6	7	8	9	10	11	12	13		
	CH <sub>3</sub> -Ser	Tyr	Ser	Met	Glu	His	Phe	Arg	Try	Gly	Lys	Pro	Val		NH <sub>2</sub>
$\beta$ -MSH (pig)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	18
	H-Asp	Glu	Gly	Pro	Tyr	Lys	Met	Glu	His	Phe	Arg	Try	Gly	Lys	Asp-OH
$\beta$ -MSH (ox)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	18
	H-Asp	Ser	Gly	Pro	Tyr	Lys	Met	Glu	His	Phe	Arg	Try	Gly	Lys	Asp-OH
$\beta$ -MSH (human)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	22
	H-Ala	Glu	Lys	Lys	Asp	Glu	Gly	Pro	Tyr	Arg	Met	Glu	His	Pro	Asp-OH



- (iv) The structure of  $\beta$ -MSH (Human) resembles  $\beta$ -MSH (pig) very closely, but has got an additional sequence of 4 amino acids towards the N-terminal end. The lysine residue in position 6 of the pig hormone is replaced by arginine in position 10 of the human  $\beta$ -MSH.
- (v)  $\beta$ -MSH (ox) differs from  $\beta$ -MSH (pig) only in position 2 where the glutamic acid residue in pig hormone is replaced by serine in the ox hormone.

It appears therefore that corticotrophin does not contain the full complement of the structural features which are necessary for MSH activity. The reduced potency of ACTH in so far as its MSH activity is concerned is probably accountable on this basis<sup>32</sup>, or it may be that the additional structural features in ACTH which are essential for its ACTH activity exerts an inhibiting influence or mask its potential MSH property<sup>37</sup>. The length of the ACTH chain does not appear to be of major importance, since an active corticotrophin molecule of 39 amino acid residues could be degraded to a product of 24 amino acid residues and yet it possesses low MSH activity<sup>39</sup>.

The most obvious difference between corticotrophin and  $\alpha$ -MSH lies in the fact that the N-terminal serine residue in the latter occurs as its N-acetyl derivative. When the N-terminal serine residue of corticotrophin was oxidised with periodate, a loss of ACTH activity with concomitant rise in MSH activity was observed<sup>33</sup>. It was also shown that when corticotrophin was subjected to mild alkaline hydrolysis which brought about specific cleavage of peptide bonds in the N-terminal 'serine-tyrosine—serine' sequence of the molecule, loss of ACTH activity is again accompanied by a significant rise of MSH activity<sup>39</sup>.

Perhaps the implication of this work will be better realized when periodate and alkali degradation products of corticotrophin are isolated and characterized. However, these studies revealed that corticotrophin could be changed into predominantly MSH-active hormone and that the N-terminal serine residues play an important role in determining which of the two activities predominate in the final molecule.

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# EFFECTS OF PROTEINS AND AMINO ACIDS ON BLOOD SUGAR LEVELS AND GLUCOSE TOLERANCE

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It was previously reported<sup>1</sup> from this laboratory that ingestion of certain proteins along with glucose profoundly modifies the glucose tolerance curve both in the normal and hyperglycaemic cases. Further results in confirmation of these observations are recorded here.

## Experimental Subjects, Materials and Methods

The subjects in the present studies were 4 normal adult males (25-45 years of age) and 2 elderly diabetics (50-55 years of age).

The proteins and protein foods used and the method employed for blood sugar estimation were the same as those described by Srinivasan<sup>1</sup>. The required dosage of protein was provided by ingesting equivalent quantities of the materials on the basis of their estimated protein content.

In the oral tolerance tests the glucose dose was 1 g/kg body-weight administered in 150-200 ml. water with a little citric acid. In the intravenous tests 50 ml of a 50 per cent sterile solution (total glucose load 25 g) was injected in 1-1½ minutes and venous blood for sugar estimation was taken at 5, 10, 15, 30 and 60 minutes thereafter.

## Results

*Oral glucose tolerance in normal subjects:* With normal subjects—normal with respect to fasting blood sugar levels and sugar tolerance—the immediate effect of protein in the oral tolerance test is positive. The general observations are that, although the glucose peak is far below the normal kidney threshold limit and blood sugar level soon returns to the original value, the shape of the curve, however, varies widely with the individual, indicating that other factors affecting glucose utilization are perhaps involved. With one subject, in the period between the ingestion of casein and the ingestion of glucose, there was no drop in the fasting blood sugar level. In two other cases, half-an-hour following the ingestion of casein there was a fall in fasting blood sugar level. In all these cases the peaks attained with casein and glucose were far less than with glucose alone.

*Intravenous glucose tolerance tests with a normal subject:* 30 g. casein was found to exert the maximum effect among the proteins and protein foods tested (Fig. 1). The effect seemed to be related to the quantity of protein ingested, for, it was less with 20 g. casein (Fig. 1). Gelatine had very little effect, the curve being almost identical with the control (Fig. 1 Curve x—x).

Vegetable proteins tried had an effect intermediate between these two extremes (Fig. 2). With tender dolichos (*Dolichos lablab*) protein, the drop in blood sugar level at



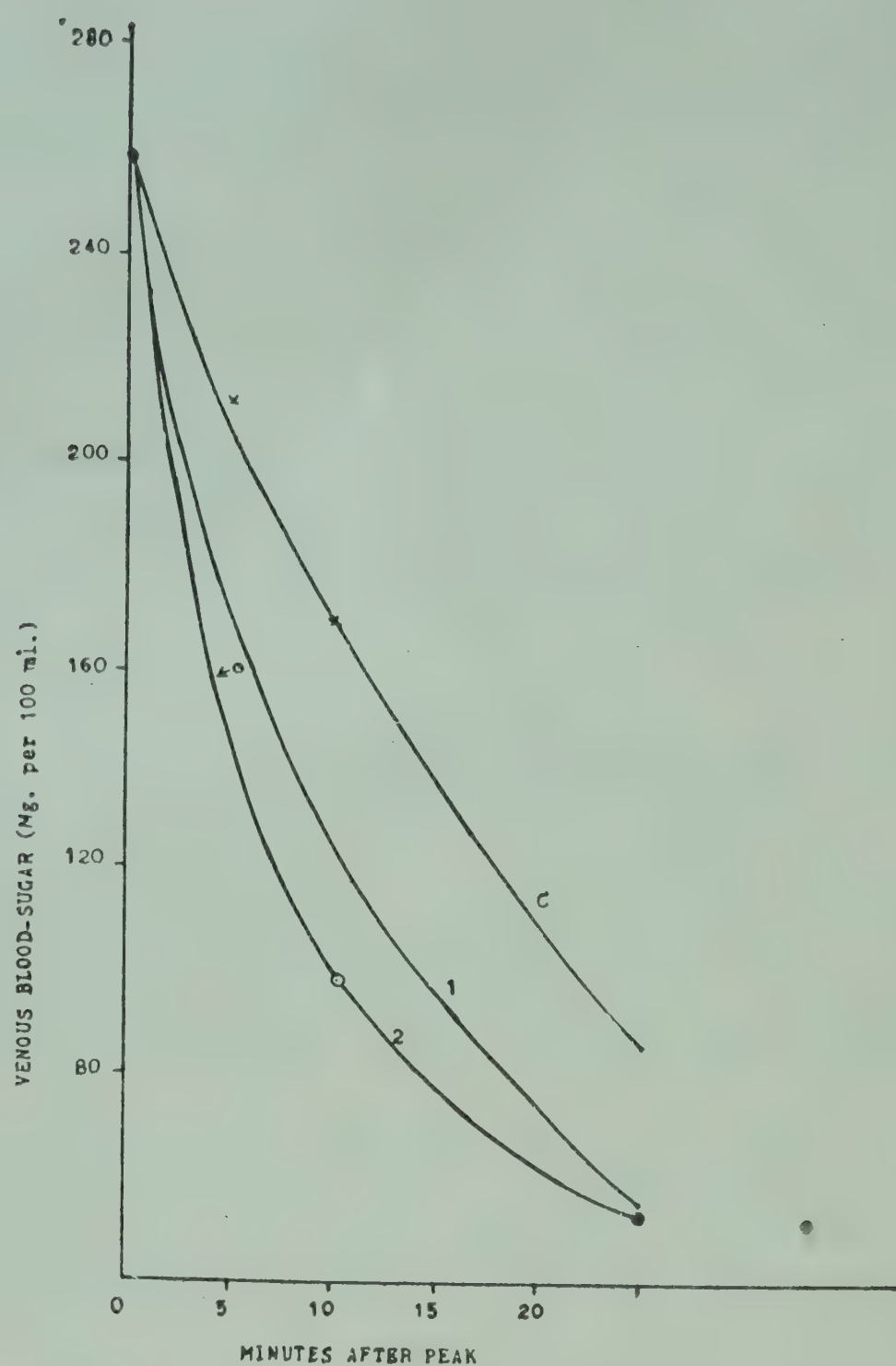


Fig. 1. Effect of casein and gelatin on intravenous glucose tolerance (i.v.g.)

C: Control, glucose only.

1: With 20 g. casein glucose.

2: With 30 g. casein glucose.

The curve in the case of gelatin (x—x) coincided with the control.

15 minutes was less than with black gram protein; but there was a steep fall to 40 mg. per cent at the end of 30 minutes (Fig. 2).

#### *Effect of amino acids on glucose tolerance*

With a view to apportioning the effect of casein among its cleavage products, amino acids were first experimented with.

A. *On intravenous glucose tolerance:* Glucogenic as well as non-glucogenic amino acids in the proportions in which they occur in casein (30 g.) were ingested severally prior to glucose in two separate intravenous glucose tolerance tests. The non-glucogenic amino acids showed a positive effect. The glucogenic amino acid mixture had no effect (the curve was the same as with the control Figure 2 A).



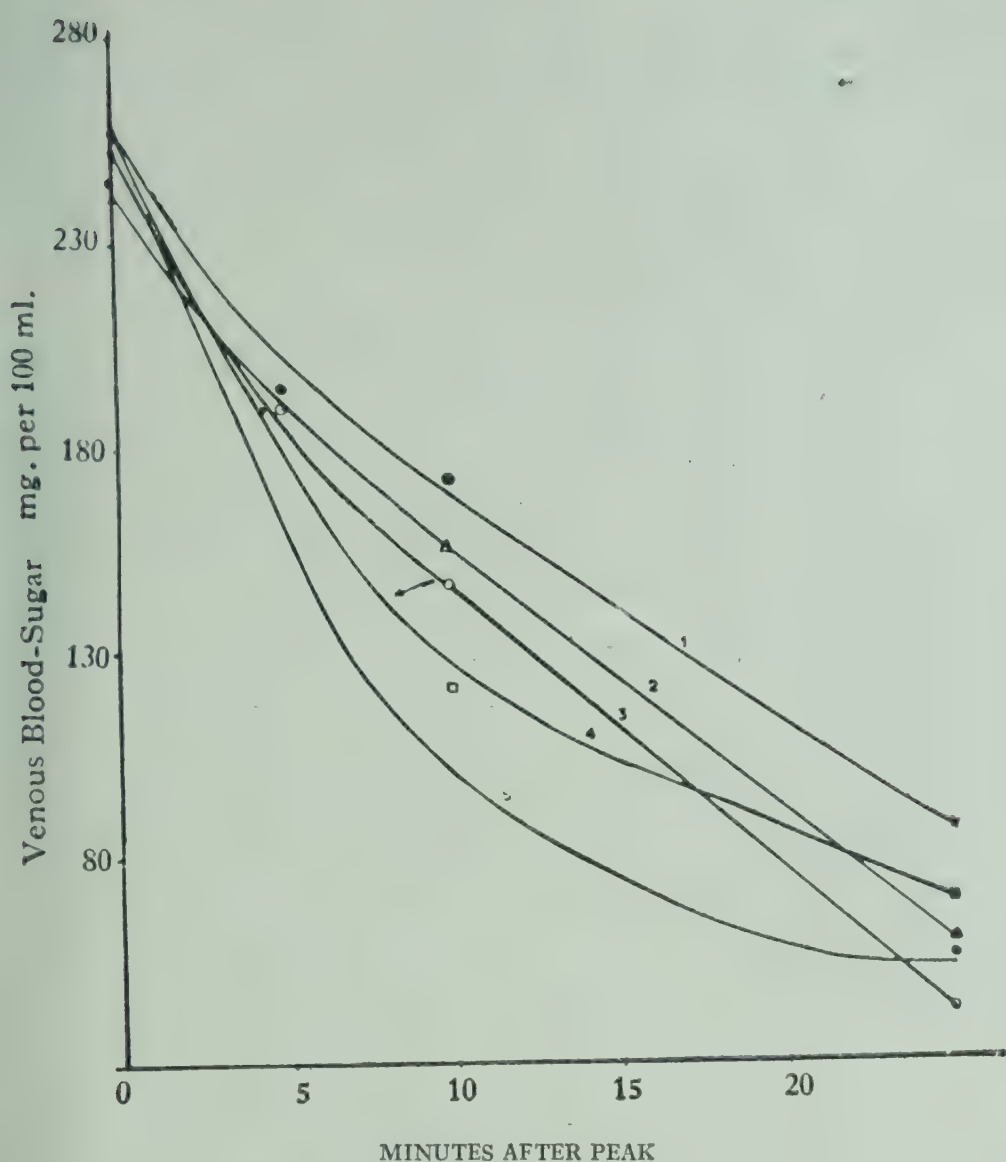


Fig. 2. Effect of vegetable proteins on i.v.g.

- 1: Control, glucose only
- 2: Black gram protein 30 g. orally and 1 hr later glucose i.v.
- 3: Dolichos protein 30 g. orally and 1 hr later glucose i.v.
- 4: Black gram 100 g. orally and 1 hr later glucose i.v.
- 5: Casein 30 g. orally and 1 hr later glucose i.v.

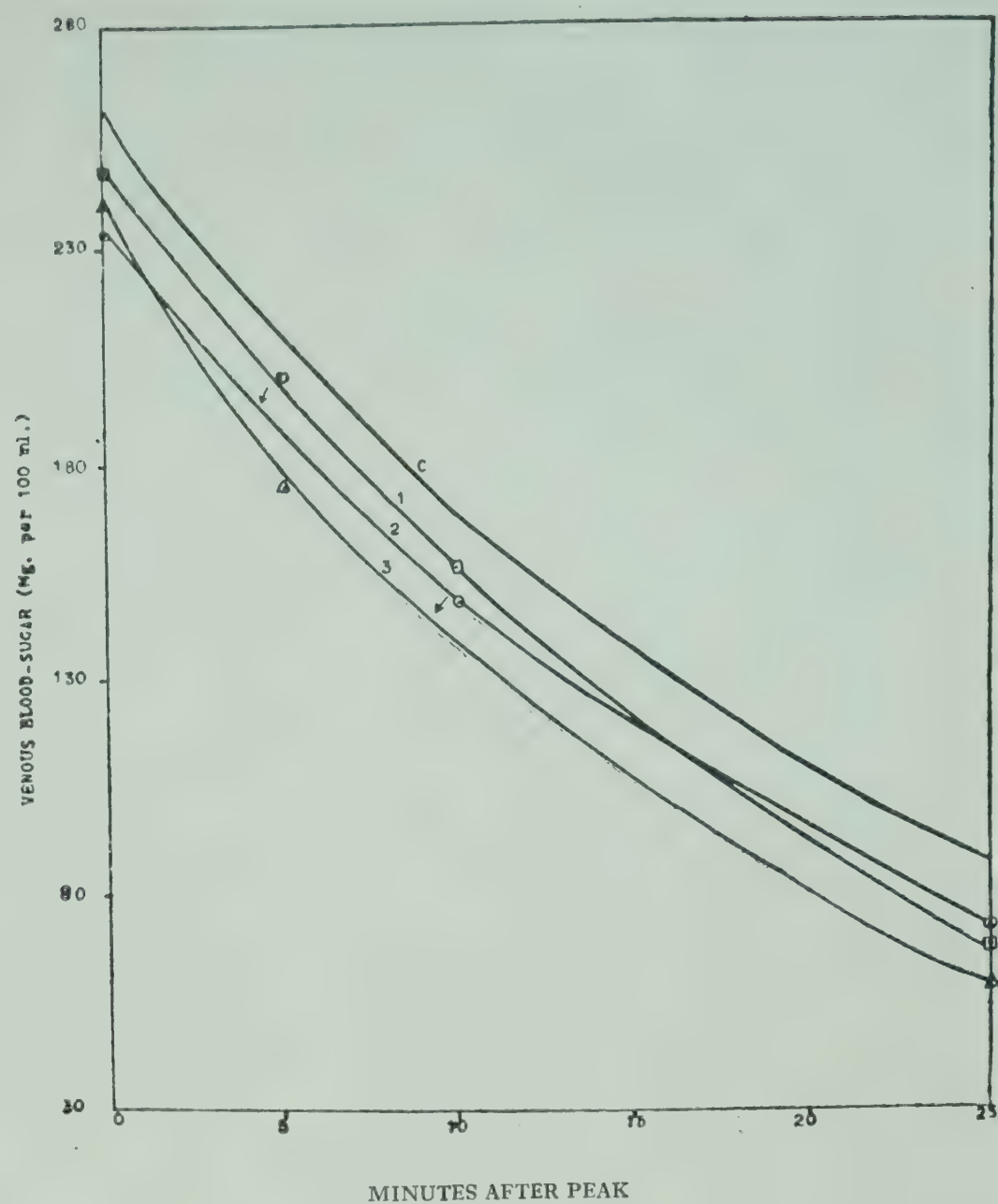


Fig. 2 A. Effect of leucine on i.v.g.

- C: Control, glucose (25 g.) i.v.
- 1: Isovaleric acid (4 g.) orally, glucose i.v.  $\frac{1}{2}$  hr later.
- 2: Non-glucogenic amino acids orally and glucose i.v.  $\frac{1}{2}$  hr later.
- 3: 4 g. DL-leucine orally and glucose i.v.  $\frac{1}{2}$  hr later.

In trials with individual amino acids the first choice was leucine since it is reported to lower blood sugar in human subjects<sup>2</sup>. DL-leucine (4 g.) produced an effect somewhat similar to the black gram protein, excepting that towards the latter half of the test, the amino acid had as good an effect as casein.

B. *On oral tolerance:* DL-leucine (4 g.) was found to have a profound effect on the oral glucose tolerance of a normal subject (Fig. 3) even better than with casein. Leucine ingested half-an-hour prior, potentiated the effect of 10 units insulin (Fig. 4) in bringing down fasting blood sugar. This effect was small where the sequence was reversed or the leucine-insulin interval was prolonged.

The effect of leucine on glucose tolerance with hyperglycaemic subjects was minimal. Also, no advantage was seen in a diabetic subject by superimposing insulin.

## Discussion

The results of intravenous tolerance tests with normal subjects show that the observed good effects of certain proteins on glucose tolerance cannot be due entirely to inter-



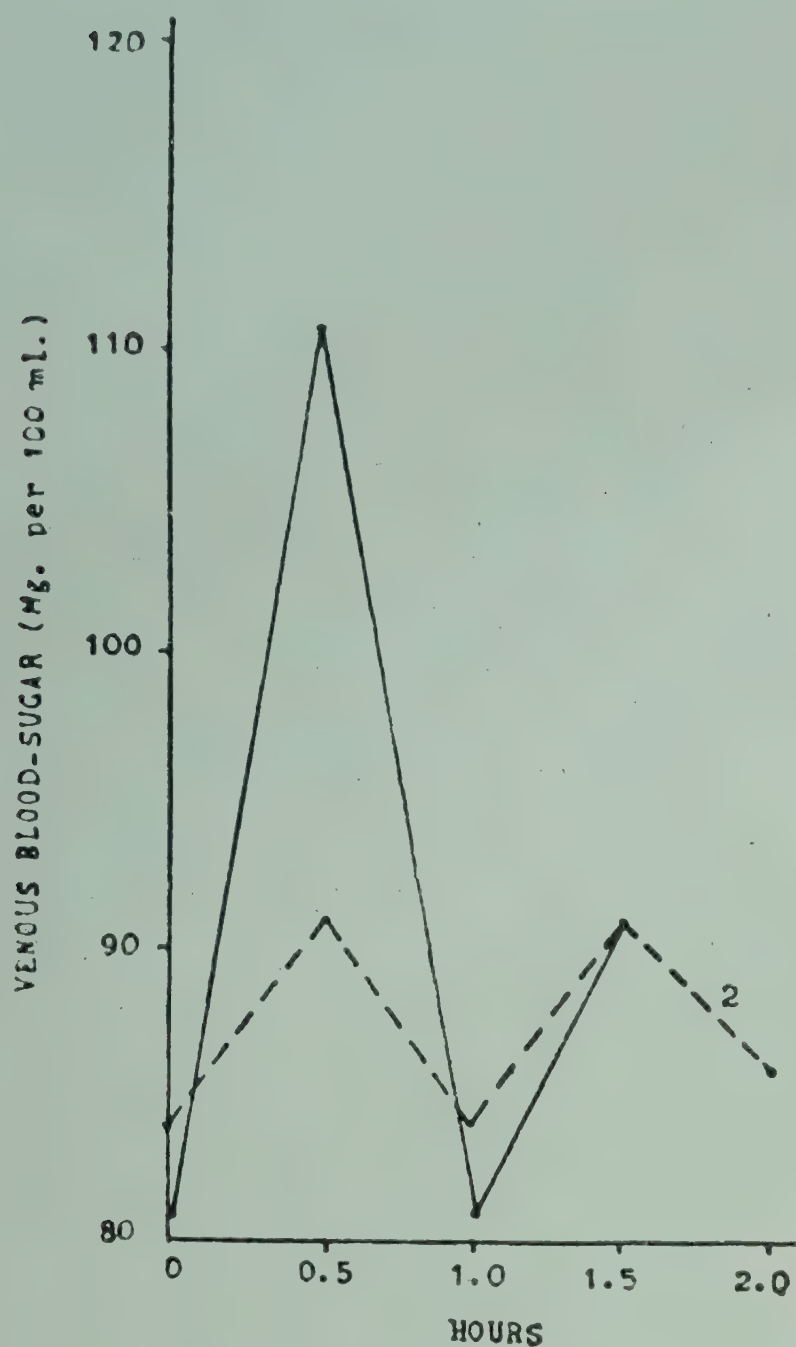


Fig. 3. Effect of leucine on oral glucose tolerance

- 1: Control, glucose only.
- 2: Glucose 4 g. dl-leucine.

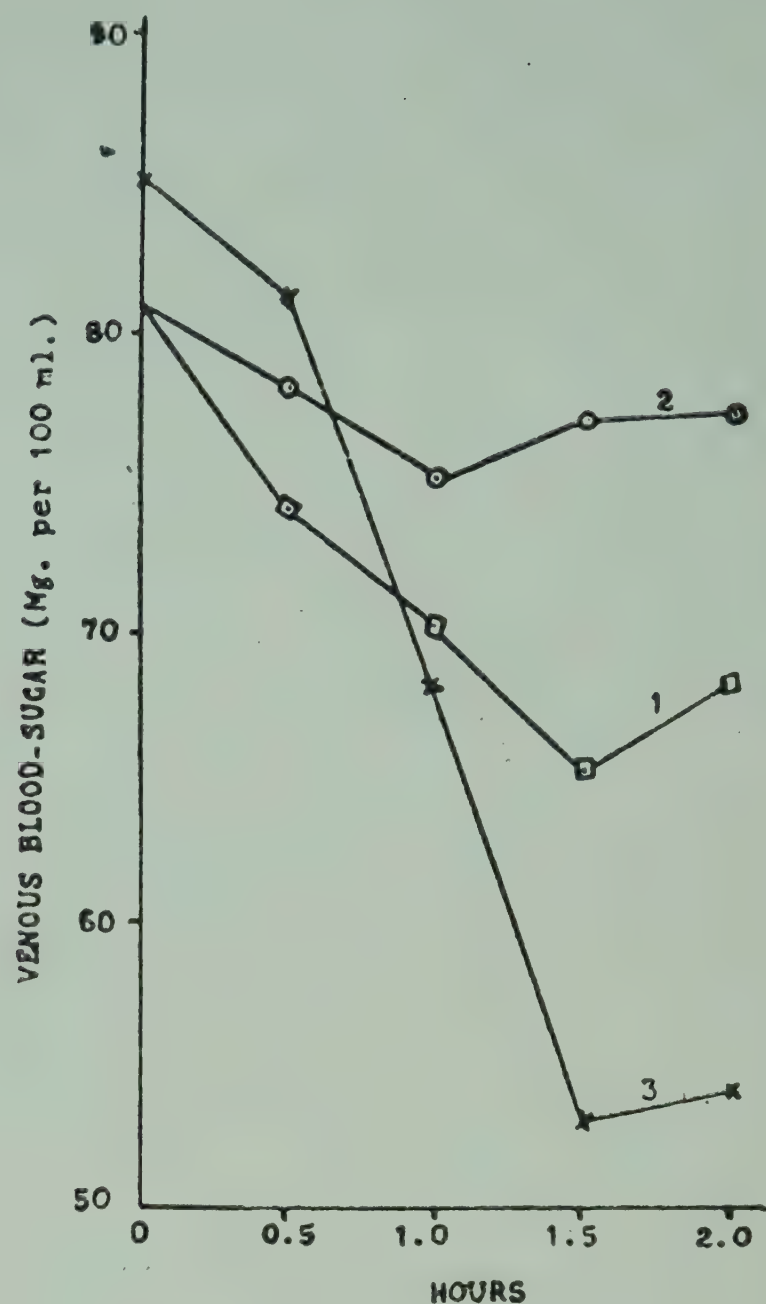


Fig. 4. Effect of leucine and/or insulin on fasting blood sugar.

- 1: 10 U insulin.
- 2: 4 g dl-leucine.
- 3: 1 and 2 together.

ference with the absorption of carbohydrate from the gut. Quite possibly, they are the effects of protein digestion products on the assimilation of carbohydrate. On this basis, the peptides or amino acids cleaved from the proteins may:

- (a) prime insulin secretion and/or potentiate insulin action (the latter through inhibition of insulinase);
- (b) inhibit glycogenolysis (by inhibiting glucose-6-phosphatase);
- (c) inhibit gluconeogenesis;
- (d) increase glycogen deposition in liver and muscles (by accelerating hexokinase activity);
- (e) independently affect other locii of intermediary metabolism of carbohydrates; and
- (f) affect other hormones.

Our preliminary observations have indicated that protein hydrolysates and amino acids do not exert any inhibitory effect on glucose-6-phosphatase. Other possible modes of action of peptides and amino acids are under study.



The specificity of proteins and certain amino acids for these effects is noteworthy. While casein is effective in glucose tolerance tests on normal or hyperglycaemic subjects, gelatine is not. This seems to be a pointer that we are dealing here with a specific effect of protein on carbohydrate metabolism, an effect which seems to vary with the *quality* of the protein.

In the hyperglycaemic cases, response of fasting blood sugar to leucine is positive with extra insulin. In normal subjects when glucose is given with leucine the peak in blood sugar level is much less than with glucose alone. Presumably, the insulin released into circulation by glucose acts together with leucine *synergistically*. It is known that glucose causes a drop in amino acid levels especially in leucine<sup>2-4</sup>. So does insulin, (over 100 units)<sup>3</sup>, but after administering insulin, if glucose is continuously infused to keep the blood sugar level above 80 mg. per cent, blood amino acid concentration does not drop<sup>5</sup>.

Leucine or casein is equally effective in oral or intravenous glucose tolerance tests in normal subjects. In oral tolerance tests with hyperglycaemic subjects, though casein and certain leguminous seed proteins produce positive effects but not gelatine, it is difficult to relate the positive protein effect to its leucine content, because of the ineffectiveness of free leucine in glucose tolerance tests with hyperglycaemic cases. The effect of casein is due, apparently, to something more than could be accounted for just by its leucine content.

Other evidence for this type of interrelationship between carbohydrate and protein metabolism has come from the few observations made under such different physiological stress conditions like hypoglycaemia<sup>6-9</sup> and kwashiorkor<sup>10</sup>. In all these cases the common feature is a lowering of blood sugar following the ingestion of protein, suggesting that this is a specific protein effect. Indeed, it has been shown that ingestion of amino acids produces a highly significant fall in blood sugar in normal subjects<sup>11</sup>.

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#### Discussion

- Q. In some of the experimental work reported from the Nutrition Research Laboratories, Hyderabad, it has been observed that during the time of active digestion and absorption of proteins after the ingestion of certain protein foods the post-prandial rises in blood sugar are comparatively low. Have any such observations been made in the course of the above work?
- A. Yes, after the ingestion of certain pulses (e.g. black gram), which contain a large amount of starch, there is a gradual fall in blood sugar of the order of 10-20 mg.



in diabetics, and, at times, in normal subjects also. Though the fall may be small, the absence of a rise is significant. A positive effect of casein has also been observed in starch-tolerance tests with diabetics.

Q. Could you ascribe the effect of the proteins to their leucine content?

A. No—and for two main reasons: (1) leucine was ineffective in diabetics who showed a favourable response to protein with regard to glucose tolerance; (2) all the leucine contained in the effective dose of protein could not be expected to be released and made available at once.

Q. What is the possible mode of action of leucine?

A. Recent reports indicate that in the hypoglycemic infants there is a substantial increase in plasma insulin-like activity after ingestion of leucine, presumably through stimulation of the pancreas. There may be other independent effects on the intermediary steps in carbohydrate metabolism. Greenstein and his collaborators observed that rats administered a  $LD_{99.9}$  dose of leucine died with severe hypoglycemia.

Q. Is there any rise in circulating insulin after protein ingestion?

A. We have just started the experimental work on this aspect.

Q. Have you made any long-term observations on the influence of protein supplements to the diabetic dietary?

A. We have made some preliminary observations on 2 or 3 diabetics whose status we could follow up over fairly long periods (4-6 months). They were taking diets tentatively formulated to include substantial supplements of casein ('Casilan', a Glaxo product) and pulses like black gram and Bengal gram. They all showed a favourable response in respect of both fasting blood-sugar levels and oral glucose tolerance.



# METABOLISM OF MUCOPOLYSACCHARIDES DURING CONNECTIVE TISSUE FORMATION

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The study of the mechanism of the formation of granulation tissue is of fundamental importance. This may be related to abnormalities in wound healing, in keloid formation and in tissue reaction to injury<sup>1</sup>.

Two of the important constituents of granulation tissue are collagen and mucopolysaccharides. Numerous *in vivo* and *in vitro* studies have been carried by various investigators<sup>2,3</sup> on the formation of collagen. However, the studies on mucopolysaccharide synthesis during granulation tissue formation have been mainly *in vivo* studies. Kodicek and Loewi<sup>4</sup> studied the uptake of S<sup>35</sup> sulphate in granulation tissue slices, obtained by tendonectomy in the guinea pig. Since tissue growth is slow and the amount of tissue was small, they were not able to study the biosynthesis of mucopolysaccharides *in situ* and their identification.

Identification of the presence of mucopolysaccharides in granulation tissues was carried out by Slack<sup>5</sup>. He used granulation tissue obtained by *in vivo* subcutaneous implantation of carrageenin in normal and scorbutic guinea pigs.

Bollet *et al*<sup>6</sup> using subcutaneous implantation of polyvinyl sponge have shown the presence of glucuronide synthesizing enzymes in this granulation tissue.

The present paper gives evidence for the presence of the enzyme systems responsible for the synthesis of 'active sulphate', the enzymatic synthesis of glucosamine-6-phosphate and also the synthesis of UDP-N-Ac glucosamine. Using granulation tissue slices, it has been possible to demonstrate the synthesis of mucopolysaccharides *in situ* in these tissues.

## Experimental Materials

Uridine diphosphoglucose (UDPG), Uridine diphospho-N-acetyl glucosamine (UDP-N-Ac-Gm), crystalline disodium adenosine triphosphate (ATP), diphosphopyridine nucleotide (DPN), crystalline glucose-6-phosphate (G-6-P) and crystalline glucose-1 phosphate (G-1-P) were obtained from Sigma Chemical Co., U.S.A. Chondroitin sulphate (CDS) and hyaluronic acid were obtained from Nutritional Biochemicals Corporation, U.S.A. Carrier-free S<sup>35</sup> sulphate and P<sup>32</sup> phosphate were obtained from Atomic Energy Commission, Trombay. Uniformly labelled glucose-C<sup>14</sup> and sodium acetate-I-C<sup>14</sup> were obtained from Radiochemical Centre, Amersham, England.

## Methods

Hyaluronidase was prepared from sheep testis and assayed as described earlier<sup>7</sup>. p<sup>32</sup> pyrophosphate (PP) was prepared by heating K<sub>2</sub> HP<sup>32</sup>O<sub>4</sub> for 1 hour at 400°C. CDS was isolated as described earlier<sup>7</sup>. Paper electrophoresis identification of mucopolysaccharides were carried out according to the method of Schiller<sup>8</sup>. Radioactivity of the isolated

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mucopolysaccharide was measured as described earlier<sup>7</sup>. Subcutaneous cotton pellet implantation technique of Meier *et al.*<sup>9</sup> was used for the induction of connective tissue formation in rats. Two months old albino rats (Indian Institute of Science, Bangalore Strain) were used. For the identification of enzymes present, 48 hour old tissues were extracted by grinding with sand and three volumes (on the weight of tissue) trihydroxy-amino methane (Tris) buffer pH 7.4 containing 0.5M KCl. The mixture was centrifuged at  $20,000 \times g$  for 20 minutes. The supernatant liquid containing 26.5 mg. per ml of protein was used in subsequent studies for enzyme activities.

The enzyme systems described in Table I were shown to be active in a granulation tissue.

TABLE I  
*Enzyme systems and their function*

Enzyme system	Reaction
1. UDPG pyrophosphorylase	$UTP + G-1-P \rightleftharpoons UDPG + PP$
2. UDPG dehydrogenase	$UDPG + 2DPN^+ \rightarrow UDPGA + 2DPNH + 2H^+$
3. Transamidase	$G-6-P + \text{Glutamine (?)} \rightarrow Gm-6-P + \text{Glutamic acid}$
4. UDPN-AcGm pyrophosphorylase	$UTP + N\text{-Ac-Gm-1-P} \rightleftharpoons UDP\text{-N-Ac-GM} + PP$
5. Active sulphate synthesizing enzyme system	$2ATP + SO_4 \rightarrow PAPS + ADP + PP$

Abbreviations : glucosamine-6-phosphate (Gm-6-P),  
N-Acetyl glucosamine-1-phosphate (N-Ac-Gm-1-P),  
UDP-glucuronic acid (UDPGA)  
3', 5'-diphosphoadenosine sulphate (PAPS).

### Assay and identification of enzyme systems

1. *UDPG pyrophosphorylase*: Presence of this enzyme in granulation tissue has been established by Bollet *et al.* This has been confirmed in the 48 hour old granulation tissue. The assay of the pyrophosphorylase was based on the disappearance of PP in the presence of UDPG.

20  $\mu$  moles of Tris buffer pH 7.4, 0.5  $\mu$  mole of UDPG, 1.0  $\mu$  mole of PP, 1  $\mu$  mole of  $MgCl_2$  and 0.1 ml. of tissue extract in a final volume of 0.25 ml. were incubated at 37°C for 30 minutes. The blank was similarly incubated without UDPG. Reaction was stopped by boiling and the disappearance of PP was measured as inorganic phosphate by adding yeast pyrophosphatase, purified according to the method of Heppel<sup>10</sup>. Inorganic phosphate was determined by the method of Fiske and Subbarow<sup>11</sup>.

2. *UDPG dehydrogenase*: Presence of this enzyme also confirms the findings of Bollet *et al.* The assay method was based on the determination of glucuronide formed by the carbazole method of Dische<sup>12</sup>.

30  $\mu$  moles of glycine buffer pH 8.7, 1.0  $\mu$  mole of UDPG, 2.5  $\mu$  moles of DPN and 0.1 ml of tissue extract in a final volume of 0.5 ml was incubated at 37°C for 50 minutes. In the blank UDPG was omitted.



3. *Transamidase*: Although Roseman and his co-workers<sup>13</sup> have shown that fructose-6-phosphate is the real substrate, it was observed that fructose-6-phosphate interferes in color formation with Ehrlich reagent. It was found that there is sufficient concentration of phosphoglucosomerase present in the tissue extract. For this reason, G-6-P was used as a substrate. The role of glutamine was not established but it was routinely used as an amino donor. Amino sugar was determined according to Blix's modification of the Elson and Morgan<sup>14</sup> method. Assay was carried out as follows:

10  $\mu$  moles of G-6-P, 20  $\mu$  moles of glutamine, 20  $\mu$  moles of tris buffer pH 7.4, 1  $\mu$  mole of versene and 0.2 ml of tissue extract in a final volume of 1 ml. were incubated at 37°C for 1 hour. The blank was run similarly except that enzyme was added after incubation. The test materials were deproteinized with trichloroacetic acid (TCA), neutralized and amino sugar was determined.

4. *UDP-N-AcGlucosamine pyrophosphorylase*: This enzyme has recently been purified from liver by Strominger and Smith<sup>15</sup>. In the present experiments, this enzyme is also found to be present in the granulation tissue extract. Using P<sup>32</sup> labelled PP and UDP-N-AcGm, formation of UTP has been demonstrated by locating the radioactive spot in the region of UTP in the paper chromatogram, using the method of Strominger and Smith<sup>15</sup>.

The assay system contained 0.2  $\mu$  mole of UDP-N-AcGm 0.8  $\mu$  moles of PP<sup>32</sup> ( $9 \times 10^5$  counts/100 sec/1  $\mu$  mole), 0.2  $\mu$  mole of MgSO<sub>4</sub>, 20  $\mu$  moles of KF and 0.05 ml. of tissue extract in a final volume of 0.15 ml. This mixture was incubated at 30°C for 30 minutes. P<sup>32</sup> labelled UTP formed was determined by adsorbing on Norit A as described by Crane and Lipmann<sup>16</sup>. In the blank, UDP-N-AcGm was omitted.

5. *Active-sulphate synthesizing system*: For identification of PAPS formed in presence of S<sup>35</sup> sulphate, the reaction mixture was adsorbed on Norit A and after washing the material was eluted from charcoal with 50 per cent alcohol containing 0.1 M ammonia. Paper electrophoresis was run parallel with known PAPS, prepared according to the method of Brunngraber<sup>17</sup>.

The radioactive spot was located at the region of known PAPS as described by Pasternak<sup>18</sup>.

The assay system consisted of 50  $\mu$  moles of imidazole buffer pH 7.0, 5  $\mu$  moles of freshly neutralized cysteine, 5  $\mu$  moles of MgCl<sub>2</sub>, 2.5  $\mu$  moles of ATP, 10  $\mu$  moles of K<sub>2</sub>S<sup>35</sup>O<sub>4</sub> ( $4 \times 10^4$  counts/100 sec/ $\mu$  mole), and 0.2 ml. of tissue extract in a final volume of 0.75 ml. Incubation was at 37°C for 1 hour. In the blank, enzyme was added at zero time. The assay was carried out by adsorbing nucleotide linked sulphate on Norit A as suggested by Bandurski and Wilson<sup>19</sup>.

The concentrations of the various enzymes described above are presented in Table II.

## Metabolism of mucopolysaccharide

1. *In vivo experiment*: 24 hours after subcutaneous cotton pellet implantation in rats 15  $\mu$ c of S<sup>35</sup> sulphate was injected intraperitoneally. After 48 hours the tissues was taken out, and the isolated mucopolysaccharide was found to contain 2,000 counts/100 sec/100 mg. of tissue. This demonstrates *in vivo* incorporation of S<sup>35</sup> sulphate into the CDS of the granulation tissue and confirms the work of Slack<sup>5</sup> who carried out similar experiments in guinea pigs.



TABLE II  
Activity of the enzymes present in granulation tissue

Enzyme	Activity/mg of protein
1. UDPG pyrophosphorylase	82 m $\mu$ moles of PP disappeared/hr.
2. UDPG dehydrogenase	42 m $\mu$ moles of UDPGA formed/hr.
3. Transamidase	0.4 $\mu$ mole of G-6-P formed/hr.
4. UDP-N-AcGm pyrophosphorylase	8 m $\mu$ moles of UTP formed/hr.
5. 'Active sulphate' enzyme system	2.5 m $\mu$ moles of PAPS formed/hr.

2. *In vitro* incorporation of  $S^{35}$  sulphate into CDS and optimum condition for maximum incorporation: The tissues formed after 48 hours cotton pellet implantation in male rats were taken for study. Slices were made and approximately 100 mg. of tissue was placed in each tube. The complete system consisted of 10  $\mu$  moles of glucose, 10  $\mu$  moles of  $MgCl_2$ , 50  $\mu$  moles phosphate buffer pH 7.4 containing 75  $\mu$  moles of NaCl and 2.5  $\mu$ c of carrier-free  $S^{35}$  sulphate in a final volume of 0.7 ml. This mixture was incubated for 3 hours at 37° C with shaking. After incubation, CDS was isolated and the radioactivity was measured. In the blank,  $S^{35}$  sulphate was added after incubation.

As indicated in Table III, glucose and  $Mg^{++}$  are required for the maximum incorporation of  $S^{35}$  sulphate into CDS.

TABLE III  
Optimum condition of the incorporation of  $S^{35}$  sulphate into CDS

Components	Counts in CDS/100 sec./100 mg of tissue
Complete system (5) ...	4,720
— Glucose (5) ...	2,380
— $Mg^{++}$ (5) ...	4,000

Figures in bracket indicate number of experiments

3. *Rate of incorporation of  $S^{35}$  sulphate with respect to the age of the tissue:* As described in Table IV, 48 hour old granulation tissue was found to have maximum activity.

4. *Sex difference in respect of rate of incorporation of  $S^{35}$  sulphate:* Data in Table V indicate that the rate of incorporation  $S^{35}$  sulphate in mucopolysaccharide is markedly different in the two sexes, the rate being twice as fast in the male rat as in the female.

5. *Incorporation of radioactive glucose- $\mu$ - $C^{14}$  and acetate- $\mu$ - $C^{14}$ :* For glucose incorporation, 1  $\mu$  mole of glucose (5  $\mu$ c/m mole), 2  $\mu$  moles of  $K_2SO_4$  70  $\mu$  moles of phosphate buffer pH 7.4, 105  $\mu$  mole of NaCl and 10  $\mu$  moles of  $MgCl_2$  were incubated with approximately 100 mg. of tissue slice (48 hour old granulation tissue), as described in Table III.



TABLE IV

*Rate of incorporation of S<sup>35</sup> sulphate with respect to age*

Experimental conditions as under Table III except that the tissue slices were obtained after varying times of implantation

Hours after cotton pellet implantation			Counts of S <sup>35</sup> sulphate incorporated/100 sec./100 mg of tissue
24 hours	(1)	...	2,660
48 hours	(5)	...	3,450 ± 150
72 hours	(5)	...	2,540 ± 200
96 hours	(5)	...	1,310 ± 120

Figures in bracket indicate number of experiments

TABLE V

*Rate of incorporation of sulphate with respect to sex*

Experimental conditions as under Table III

Source			Counts/100 sec./100 mg tissue
Male rat	(5)	...	4,170 ± 500
Female rat	(5)	...	2,028 ± 400

Figures in bracket indicate number of experiments

For acetate incorporation, 2.2  $\mu$  moles of acetate (1  $\mu$ c/1.1  $\mu$  mole), 2  $\mu$  moles of K<sub>2</sub>SO<sub>4</sub>, 10  $\mu$  moles of glucose, 70  $\mu$  moles of potassium phosphate buffer pH 7.4, 80  $\mu$  moles of KCl and 10  $\mu$  moles of MgCl<sub>2</sub> were incubated as described earlier.

It was found that both glucose and acetate were incorporated into mucopolysaccharide. The results are presented in Table VI. It is to be noted that the sex difference is quite similar to that of sulphate incorporation. Isolated mucopolysaccharide was found to be completely digestible with hyaluronidase. Paper electrophoresis indicates the presence of radioactivity both in hyaluronic acid region and CDS region.

TABLE VI

*Incorporation of glucose and acetate into mucopolysaccharides*

Source of tissue			Counts/100 sec./100 mg tissue	
			With C <sup>14</sup> Glucose	With C <sup>14</sup> Acetate
Male rat	(2)	...	3,000	...
Female rat	(2)	...	1,610	1,040

Figures in bracket indicate number of experiments



## Discussion

The tissue extracts from 48 hours old granulation tissue were found to contain the enzyme system involved in the glucuronide synthesis. This confirms the findings of Bollet *et al.*, that the glucuronide enzyme system is present in these tissues. The present investigation has been further extended to show the presence of the enzyme system responsible for the synthesis of 'active sulphate', glucosamine-6-phosphate and uridine diphospho-N-acetyl glucosamine. All these compounds are known to be precursors for the synthesis of hyaluronic acid and chondroitin sulphate<sup>20,21</sup>. It is to be noted that all these enzymes are present at a stage when there is rapid synthesis of mucopolysaccharides.

Studies on sulphate incorporation into CDS indicate that it is very rapid at the initial stage of granulation. However, Suzuki and Strominger<sup>22</sup> have pointed out in their investigation with hen oviduct that sulphate may be incorporated even at the level of CDS. This report suggests that mere S<sup>35</sup> sulphate incorporation is not an indication of CDS synthesis. In the present study it has been shown that glucose and acetate are also incorporated into mucopolysaccharides. The incorporation of glucose and acetate and the presence of various enzyme systems which are essential for the synthesis of mucopolysaccharide strongly suggest that the synthesis of mucopolysaccharide is occurring at the monosaccharide level *in situ*. Paper electrophoresis and hyaluronidase treatment on the isolated mucopolysaccharides suggest that both hyaluronic acid and CDS are synthesized.

The tissue slice experiments indicate a pronounced sex difference in relation to the rate of synthesis of mucopolysaccharides. These observations are based on wet weight of tissue. On this basis the rate of synthesis of mucopolysaccharides is about twice as fast in the male rat as in the female. Boucek *et al*<sup>3</sup> have found that the rate of incorporation of proline and lysine into hydroxyproline and hydroxylysine of the collagen of the granuloma shows a similar sex difference. This can be explained on the basis of the present work, since mucopolysaccharide synthesis precedes collagen fibril formation.

## Summary

Enzyme systems responsible for the synthesis of uridine diphosphoglucose, uridine diphosphoglucuronic acid, glucosamine-6-phosphate, uridine diphospho-N-acetyl glucosamine and 3',5'-diphosphoadenosine sulphate are shown to be present in granulation tissue.

Tissue slice experiments show the formation of mucopolysaccharides in granulation tissue. Evidence is presented to show that mucopolysaccharides are synthesized in these tissues *in situ*.

The rate of synthesis of mucopolysaccharides is shown to be twice as fast in male rats as in the female.

## Acknowledgment

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## Discussion

- Q. Could the observed sex differences in the rates of synthesis of mucopolysaccharides *in vitro* be ascribed to hormonal effects?
- A. There is no direct evidence for this. If it is so, then the effects have to be reckoned as the long-term effects of hormones since they are observed in tissue slices *in vitro*.
- Q. In the formation of myelin in nerve tissue, is protein formed first or mucopolysaccharide?
- A. The mucopolysaccharide is formed first and later the protein.
- Q. Vitamin A is known to play an important part in mucoprotein formation. Is it concerned with the synthesis of polysaccharide or protein?
- A. It is primarily involved in the formation of the mucopolysaccharide moiety.



# IMMUNOCHEMICAL STUDIES ON THE PROTEINS OF *VIBRIO CHOLERAE*

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In comparison with the extensive literature available on the immunochemistry of *Vibrio cholerae* polysaccharides, information on the protein antigens of cholera and non-cholera vibrios is rather scanty.

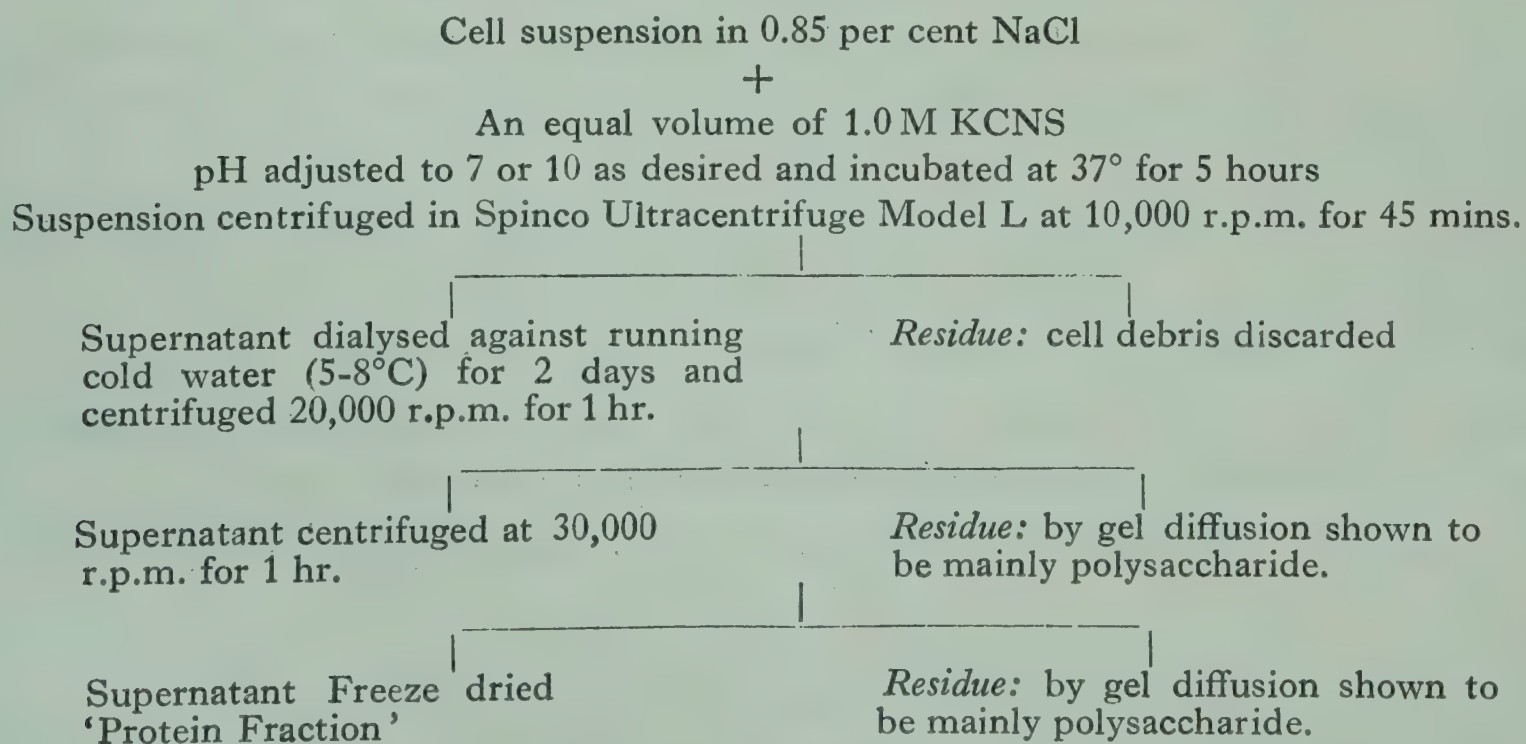
Various protein fractions have been reported from time to time, but their relationship to one another is not known<sup>1-2</sup>. Their localization and also their importance in the antigenicity and virulence of *V. cholerae* are not clear. It was, therefore, considered to be of interest to make a systematic investigation of the protein antigen present in the vibrio cells and the observations made in a preliminary study are reported in the present communication.

## Experimental

Most of these studies were made using *V. cholerae*, Ogawa 4. Sixteen to eighteen hours growth on agar in roll bottles was harvested with cold 0.85 per cent sodium chloride, cells collected by centrifugation in a Sharples super centrifuge and washed twice by suspending in 0.85 per cent sodium chloride and recentrifuging.

The protein antigens were isolated from the cells, using potassium sulphocyanide as in the case of *P. pestis*.<sup>13</sup> The fractionation schedule routinely adopted is outlined schematically in Fig. 1.

Fig. 1. Fractionation of *V. cholerae* cells for the isolation of protein antigens.



Nitrogen was estimated by the micro-Kjeldahl procedure<sup>14</sup> and phosphorus according to Fiske and Subbarow as modified by Sumner<sup>15</sup>. For gel diffusion analysis of the antigens the Ouchterlony plate method was employed.

Precipitin sera were prepared by injecting rabbits on alternate days with live culture suspensions (3000 million ml) in gradually increasing doses from 0.25 to 2.0 ml. In all, about 15 injections were needed to get a potent antiserum.



Descending chromatography was performed on sheets of Whatman No. 1 employing butanol: benzene: pyridine: water (5:1:3:3) as developing solvent, identifying amino acids and amino sugars by ninhydrin and sugars by aniline hydrogen phthalate. Paper electrophoresis was carried out in a model 5K-51, Kelab A-B- apparatus employing phosphate buffer (ionic strength M/15) pH 8.0 (Voltage 250 and 20 ma current). Calcium phosphate gel for adsorption was prepared according to Keilin and Hartree<sup>16</sup>.

## Results

*Chemical composition and properties:* The proteins isolated at pH 10 and 7 had respectively 10 and 7 per cent N, no phosphorus and were strongly biuret positive and Molisch negative. The absorption spectrum of the fractions showed max at 280 m $\mu$  and no hump or inflexion at 260 m $\mu$ , indicating that they were mainly protein in nature, free from bound nucleic acids or nucleotides. In phosphate buffer of ionic strength M/15 and pH 8.0, the fractions moved towards the anode. Paper chromatography of 6N HCl hydrolysates of one of the antigens revealed the presence of aspartic and glutamic acids, serine, alanine, tyrosine, glucosamine, galactosamine and a few other unidentified ninhydrin positive spots.

*Gel diffusion analysis:* Diffusion analysis of protein fractions prepared at pH 7, 8, 9 and 10 was carried out in Ouchterlony plates. The two zones near the anti-serum pool observed in pH 7 plate gradually broke up into 3 or more, as pH shifted towards 10. The third zone started appearing at pH 8 and became most intense at pH 10. In addition to these, the plates showed up a faint zone corresponding to polysaccharides<sup>10</sup> after several days of incubation.

*Heat stability of the antigens:* The antigen zone nearest to the anti-serum pool could not be demonstrated with heated extracts. Heating the antigens at 100° for 20 min. completely destroyed the first two antigens, whereas the antigen 3 which appeared in pH 10 extracts was comparatively heat stable.

*Removal of polysaccharide:* Centrifugation at 46,900 g in the Spinco Ultracentrifuge Model L was found to effect a separation of the polysaccharide from the protein antigens. The sedimented fraction gave a single prominent zone corresponding to the polysaccharide in gel plates.

*Adsorption by calcium phosphate gel:* Attempts were made to purify the protein isolates obtained from KCNS extracts by adsorption on calcium phosphate gel. The precipitin activity could be completely adsorbed on calcium phosphate gel. On elution of the gel with M/5 phosphate buffer and dialysis, most of the antigenic activity could be recovered in the eluate. Further elution with M/2 phosphate buffer did not extract any more of the activity. Since the phosphate eluates did not show up a zone corresponding to polysaccharide, it would appear that the latter is firmly adsorbed on the calcium phosphate gel.

*Cross reaction pattern:* Cross reaction patterns of the protein and polysaccharide antigens with Ogawa and Inaba precipitin sera are presented below:

Reactant	Precipitin antisera	
	Ogawa	Inaba
Ogawa polysaccharide ... ..	+	+
Inaba       ,,       ... ..	—	+
Ogawa protein antigen       ... ..	+	+
Inaba       ,,       ,,       ... ..	+	+



It is clear from the above that, unlike the polysaccharide, the protein antigen appears to be non-specific in serological reactions.

### Summary and Conclusions

This preliminary study reveals the potentialities of ultracentrifugation and calcium gel adsorption in the separation and purification of the non-specific protein antigens of *V. cholerae*. Paper electrophoresis has indicated that the protein fractions isolated are presumably homogeneous in nature. This can be verified only by further purification and also by employing independent criteria of assessment of homogeneity, such as immuno-electrophoresis or ion-exchange chromatography. Whether the heat labile antigen is associated with any enzymic activity is also open to further investigation. Localization of the protein antigens on the different anatomical structures of the cell is being presently attempted by controlled lysis of the cells by versene and lysozyme. If by a combination of conventional methods of protein fractionation, such as the ammonium sulphate precipitation with gel adsorption or ion-exchange chromatography, one could obtain integrally homogeneous protein fractions from different sub-types of the organism, it would go a long way in elucidating the specificity or otherwise of protein antigens in relation to their chemical properties.

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# STUDIES ON THE CHARACTERIZATION AND THE MECHANISM OF FORMATION OF AN EXO-ENZYME PROTEIN (LECITHINASE) IN *VIBRIO EL TOR*

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*Vibrio cholerae* including *Vibrio El Tor* were found to excrete lecithinase in their culture medium<sup>1-3</sup>, but the exact nature of the enzyme was not properly studied. The present work is concerned with the characterization of this enzyme elaborated by the organism *Vibrio El Tor*, which has been found to excrete the enzyme in quantities greater than that by other *Vibrio* species. Some of the properties of this enzyme also have been presented here.

Preliminary investigations have been made on the mechanism of excretion of this enzyme by the resting cell suspension in relation to optimum pH of excretion and also to some other factors such as the effect of sugars and inhibitors.

## Experimental Materials and Methods

*Substrates: Owolecithin:* Prepared according to the method of Pangborn<sup>4</sup> and purified by column chromatography<sup>5</sup>. *Lysolecithin:* Prepared according to the method of Hanahan<sup>6</sup>.

*Enzyme preparation:* The organism *Vibrio El Tor* was grown aerobically in surface cultures using peptone-water medium for 48 hours and the medium was centrifuged at 4000 r.p.m. for 30 mins at 0°C. The clear centrifugate was taken and filtered through bacterial filter. It was then fractionated with ammonium sulfate and the precipitate obtained between 40-75 per cent saturation was collected. This was dissolved in M/100 phosphate buffer and dialysed against M/100 phosphate buffer and kept frozen at -15°C. for about four weeks up to which no loss of enzyme activity was noticed.

*Incubation:* Incubations were carried out in 10 ml stoppered centrifuge tubes into which the requisite amount of lipid substrate in chloroform solution (0.75  $\mu$  mole) was added. The solvent was removed *in vacuo* at about 40°C and the lipid was then emulsified in buffer solution (50  $\mu$  mole barbital buffer pH 8.0, unless otherwise specified). The enzyme equivalent to approximately 0.15 mg protein was then added in a total volume of 1 ml of reaction mixture and incubated at 37°C, usually for 60 minutes. The reaction was stopped by the addition of trichloroacetic acid solution to a final concentration of 5 per cent, in the presence of bovine serum albumin (5 mg per tube) which was used to co-precipitate the unreacted lipid substrate. This was then centrifuged, the centrifugate was extracted once with three volumes of a mixture of chloroform and isobutanol (2:1, v/v) and was again centrifuged. The aqueous portion was then separated and analysed for the reaction products. Chromatographic detection of the reaction products was done



according to the method described by Dawson and Rowlands<sup>7</sup> and the presence of glycerophosphorylcholine was established.

*Estimations:* Choline was estimated by the periodide method described by Appleton *et al.*<sup>8</sup> Phosphorus was estimated according to the method of Fiske and Subbarow<sup>9</sup>. The fatty acyl ester groups were determined by the method described by Snyder and Stephens<sup>10</sup>. Glycerophosphoryl choline was assayed by estimating the water soluble choline before and after hydrolysis in normal HCl at 100°C for 20 minutes<sup>11</sup>.

*Enzyme excretion by the resting cell suspension:* 1 ml suspension of *Vibrio El Tor* in saline containing approximately 20 mg cells, on dry weight basis, was incubated with a mixture of buffer and other reagents used, if any, for 16 hours. The total volume of the reaction mixture was kept 10 ml, the amount of buffer used being 100  $\mu$  moles. After the incubation period, the reaction mixture was centrifuged at 15,000 g for 15 minutes at 0°C and the clear centrifugate was assayed for the enzyme activity according to the method described previously. The degree of lysis of the organism was estimated by measuring the turbidity of the solution in a Klett-Summerson photoelectric colorimeter using 540  $m\mu$  filter. The protein excreted was estimated according to the biuret method<sup>12</sup> or by the use of the Lowry reagent<sup>13</sup>. The excretion of substances having absorption maxima at 260  $m\mu$  was also estimated in a Beckman DU Spectrophotometer using 1 cm cell.

## Results and Discussion

*Nature of the lecithinase:* The enzyme attacks only lysolecithin with the simultaneous liberation of water soluble phosphate ester containing choline and the constituent fatty acid. It has no action on purified ovoidlecithin. The aqueous portion of the incubation mixture was analysed for the water soluble choline compound and phosphorus. It was found that, after the enzyme reaction, there is an increase in the water soluble choline compound in quantitative proportion to increase in the water soluble phosphorus and there is no liberation of free choline. Consequently, experiments were done in which the disappearance of acyl fatty ester bonds was compared with the appearance of acid soluble phosphorus. Chromatographic studies with the aqueous portion of the reaction mixture indicated the liberation of glycerophosphoryl choline from lysolecithin by this enzyme. The enzyme is, therefore, regarded as Phospholipase B.

*Excretion of the enzyme during the growth of the organism:* The growth of the organism *vs.* the liberation of the enzyme has been studied in detail. It has been found that the excretion of the enzyme rises to a maximum just at the stationary phase (48 hrs) after which both the growth and the excretion of enzyme remain constant up to 72 hours (Table I). The incubations were done according to the method described previously.

*Optimum pH:* The optimum pH of the enzyme is 8.0 and the enzyme is completely inactivated at pH 3.1. Citrate-phosphate, phosphate and barbital buffers (0.1 M) were used. The pancreas phospholipase B has a pH optimum at 6.0, whereas the *Penicillium notatum* phospholipase B is optimally active at pH 3.3.<sup>15</sup> In this respect, however, these enzymes are not similar.



TABLE I

Hr.	Growth Klett reading at 540 m $\mu$	$\mu$ mole GPC liberated/ml culture filtrate/hr
5	40	0
8	125	0
11	164	0.03
14	190	0.09
18	215	0.26
24	230	0.54
42	275	1.10
48	278	1.70
72	275	1.75

*Heat stability:* The enzyme is not very much susceptible to heat and retains about 50 per cent of its activity even after heating at 100°C for 5 minutes, but loses its activity completely after heating for 15 minutes at 100°C. The enzyme, therefore, is not similar to the *Pencillium notatum* phospholipase B which is destroyed completely on heating at 61°C for 15 minutes<sup>15</sup>. *Vibrio El Tor* phospholipase B is, therefore, more stable towards heat.

*Effect of metal ions:* Using Cu<sup>++</sup>, Mn<sup>++</sup>, Zn<sup>++</sup> and Ca<sup>++</sup> (10<sup>-3</sup>M), it was found that Zn<sup>++</sup> inhibits the enzyme to an appreciable extent, whereas the per cent inhibition due to Cu<sup>++</sup> is very low (not above 5). This is quite interesting since it has been found that pancreas phospholipase B is stimulated by Ca<sup>++</sup> in the case of monophosphoinositide as substrate, while in the case of lysolecithin as substrate there is no effect<sup>14</sup>. Ca<sup>++</sup> was found to have no effect on the *P. notatum* phospholipase either when it was attacking lysolecithin or monophosphoinositide. Fairbairn<sup>15</sup> noticed that Cu<sup>++</sup> at a concentration of 10<sup>-2</sup> molarity caused about 40 per cent inhibition of *P. notatum* phospholipase B. The inhibitory effect of Zn<sup>++</sup> on the *Vibrio El Tor* phospholipase B is quite appreciable.

*Effect of inhibitors:* Among the inhibitors tested NaF, azaguanine, cyanide and chlormphenicol (10<sup>-3</sup>M) had no effect on the enzyme. DNP brought about 20 per cent inhibition, while NaF (10<sup>-2</sup>M) and PCMB (10<sup>-3</sup>M) were quite powerful in inhibiting the enzyme and caused about 45 per cent inhibition. With versene, the inhibition was 40 per cent and with azide, about 15 per cent. The inhibition caused by PCMB and versene was fully reversed by the addition of glutathione and Ca<sup>++</sup> respectively. In all these cases, the enzyme was preincubated with the inhibitors for 15 minutes.

The enzyme is similar to *P. notatum* phospholipase B with respect to the effect of KCN<sup>14</sup>. It has been found by Dawson<sup>14,16</sup> that NaF is a potent inhibitor of pancreas phospholipase B and it also appreciably inhibits *P. notatum* phospholipase. In this respect all these enzymes behave similarly. Inhibition by PCMB and its simultaneous reversal by glutathione indicates that the enzyme contains sulfhydryl groups which are essential for its activity.



*Effect of increasing substrate concentration:* The effect of increasing substrate concentration on the rate of enzyme reaction is presented in Table II. The incubations were done according to the method described previously.

*Reaction kinetics:* The reaction kinetics of the enzyme using 0.75  $\mu$  mole substrate and enzyme equivalent to 0.14 mg protein has been presented in Table III.

TABLE II

Substrate concn. (Lysolecithin) $\mu$ mole	GPC liberated, $\mu$ mole
0.25	0.087
0.50	0.172
0.75	0.230
1.00	0.258
1.50	0.283
2.00	0.280

TABLE III

Time in minutes	GPC liberated, $\mu$ mole
0	0
5	0
15	0.10
35	0.15
65	0.25
120	0.35

*Isolation of the enzyme:* The enzyme was isolated from the culture filtrate by ammonium sulfate fractionation and was almost completely precipitated between 40-75 per cent saturation. The fraction obtained between 0-40 per cent saturation contained very little activity.

*Studies on the mechanism of excretion of enzyme by the resting cell suspension:* Preliminary studies indicated that neutral pH (7.2) was best for the optimum excretion of the enzyme. Correlation between the excretion of protein and enzyme with respect to pH was not clear (Table IV).

Buffer composition had no effect on enzyme excretion and the degree of lysis (Table V) in contradiction to the findings of Krishnamurti and Sen Gupta<sup>17</sup> that the degree of lysis of *Vibrio cholerae* by buffers in the presence of versene varies with respect to buffer composition.



TABLE IV

Buffers (0.1 M)	Protein mg/ml	$\mu$ mole GPC liberated per ml/2 hr
Citrate-phosphate, pH 3.0 ...	0.02	0
Acetate, pH 5.1 ...	0.30	0.275
Phosphate, pH 7.2 ...	0.35	1.630
Barbital, pH 8.3 ...	0.30	1.400

TABLE V

Buffers used	Protein mg/ml	$\mu$ mole GPC liberated/ml per 2 hr	Per cent lysis
Phosphate, pH 7.2 ...	0.35	1.50	53
Tris, pH 7.2 ...	0.35	1.55	56
Barbital, pH 7.2 ...	0.37	1.60	53

Sugars have a profound effect on the synthesis of enzyme and the nature of the sugars accelerating or inhibiting the formation of enzyme depend on the extent to which they are utilized by the organism<sup>18</sup>. A correlation was observed between the utilization of different sugars by *Vibrio El Tor* with respect to the excretion of the enzyme (Table VI). Also sugars which are slightly utilised (lactose and arabinose) caused excretion of more enzyme than those readily utilised (glucose, fructose and galactose). Glucose, fructose and galactose, however, considerably inhibited excretion but no clear-cut correlation amongst these three sugars emerged. There was little correlation between the amount of enzyme excreted and the degree of lysis of cells, the two processes being almost independent of each other. It was observed that among the various inhibitors examined, DNP and also chlor-

TABLE VI

Sugar used	$\mu$ l. O <sub>2</sub> uptake per mg cell (dry wt. basis) per hr*	$\mu$ mole GPC liberated/ml per 2 hr	Protein mg/ml	Per cent lysis
Glucose ...	72.3	0.60	0.46	42
Fructose ...	39.6	0.45	0.34	28
Galactose ...	14.2	0.39	0.34	42
Lactose ...	9.4	1.15	0.34	35
Arabinose ...	4.3	2.00	0.37	35
Control ...	...	1.50	0.33	53

\* Endogenous value was deducted in all the cases.



amphenicol caused about 20 per cent inhibition in enzyme excretion while azaguanine had apparently no effect.

Using buffers of different composition and of varying pH it was found that, under optimum conditions of enzyme excretion, there was an increased excretion of substances having absorption maxima at 260 m $\mu$ , suggesting a direct correlation between the enzyme excretion and the nucleic acid pool of *Vibrio El Tor*.

## Summary

1. *Vibrio El Tor* excretes a lecithinase in its culture filtrate which has been characterized as phospholipase B.
2. The optimum pH of this enzyme is 8.0.
3. The enzyme is insensitive to metal ions such as Cu<sup>++</sup>, Ca<sup>++</sup> and Mn<sup>++</sup>, but Zn<sup>++</sup> causes about 40 per cent inhibition of its activity.
4. Inhibitors such as KCN, azaguanine and chloramphenicol have no effect, and azide and DNP inhibit the activity to the extent of 15 and 20 per cent respectively, whereas inhibition by PCMB, NaF and versene ranges from 40-45 per cent. The inhibition by versene is reversed by Ca<sup>++</sup>, while that by PCMB is reversed by glutathione.
5. Effect of increasing substrate concentration and the reaction kinetics have been studied.
6. The organism excretes the enzyme optimally at pH 7.2; at pH 3.0 no excretion is noticed.
7. In the presence of different buffers of the same pH, the organism excretes the same amount of enzyme and the degree of lysis also does not vary with different buffers.
8. Sugars which are utilized by the organism rapidly (glucose, fructose and galactose) usually inhibit the enzyme excretion, whereas those which are not easily utilized (lactose and arabinose) may even stimulate the same.
9. Effect of inhibitors on the excretion of enzyme has also been studied.

## Acknowledgment

The authors express their sincere thanks to Prof. B. C. Guha for his valuable advice and interest in this work. Thanks are also due to Dr R. M. C. Dawson, Institute of Animal Physiology, Cambridge, for his kind gift of an authentic sample of GPC required for chromatographic detection and to the Indian Council of Medical Research for financial help.

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### Discussion

- Q. Do you implicate lecithinase in the hemolytic activity?
- A. There is some evidence for this. At any rate, lecithinase activity is positively correlated with pathogenicity.
- Q. Vibrios are known not to utilize lactose or galactose. What was the idea in examining the effect of these sugars? Did you obtain a strain which utilized them?
- A. The experiments reported here show that the strain we used also does not utilize lactose but utilizes galactose to some extent. Still they show contrary effects on enzyme excretion. This deserves to be carefully investigated further.
- Q. Was the effect of  $Mn^{++}$  ions at higher concentrations studied?
- A. No.

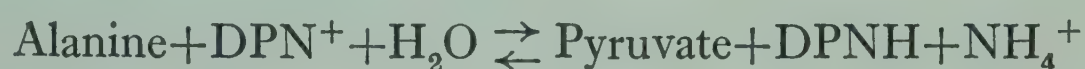


# ALANINE DEHYDROGENASE IN MYCOBACTERIA

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The only amino acid dehydrogenase that has been purified and studied so far is the DPN-specific glutamic dehydrogenase<sup>2</sup>. Recently, the reductive amination of pyruvate to alanine by cell-free extracts of *Bacillus subtilis*<sup>1</sup> and by *Mycobacterium tuberculosis* H<sub>37</sub> Ra<sup>3</sup> have been reported. This alanine dehydrogenase has been shown to be DPN-specific and catalyses the reversible reaction:



The reaction has been shown to take place in the absence of even a catalytic amount of any linked system reported by Kritzman<sup>4</sup>, where an alanine-oxalacetate trans aminase is shown to couple with the amination of pyruvate, or that of Braunstein<sup>5</sup> and Nisman<sup>6</sup> where the presence of glutamic dehydrogenase in association with a glutamic-pyruvate transaminase could give an overall reaction of alanine dehydrogenase.

In view of the lack of data on the occurrence of the alanine dehydrogenase in other strains of Mycobacteria, particularly the virulent strain H<sub>37</sub> RV, whose amino acid metabolism is under study in our laboratory, investigations on alanine dehydrogenase in a few strains of Mycobacteria have been undertaken. This paper describes some of these studies.

## Experimental Materials and Methods

*Organisms used:* *Mycobacterium tuberculosis var-hominis* H<sub>37</sub> RV and H<sub>37</sub> Ra strains, *Myco-lacticola*, *Myco-phlei* and *Myco-smegmatis* were used. Stock cultures of the *Myco-tuberculosis* strains were maintained on Patrick's<sup>7</sup> media with bimonthly transfer to fresh media. The other strains were maintained on nutrient agar with biweekly transfer to fresh media. For experimental purpose, all strains were grown on liquid synthetic medium containing ammonium sulphate as the source of nitrogen, with the following media composition<sup>8</sup>.

Ammonium sulphate	2.5 g
KH <sub>2</sub> PO <sub>4</sub>	5.9 g
Mg citrate	1.5 g
K <sub>2</sub> SO <sub>4</sub>	0.5 g
Glycerol	20 ml
Water to make up to	1.000 ml, pH 7.2

Amino acids used were obtained from Hoffman-La-Roche; DPN, TPN and FAD from Sigma Chemical Company; sodium pyruvate from E. Merck & Co.; and Tris [tris (hydroxy methyl) amino methane] from Light & Co.

DPN and DPNH denotes oxidised and reduced forms of diphosphopyridine nucleotide. TPN=Oxidised form of triphosphopyridine nucleotide, FAD=Flavine adenine dinucleotide. Tris=Tris (hydroxy methyl) amino methane.



*Preparation of cell-free extract:* Surface cultures of *M-tuberculosis* H<sub>37</sub> RV, 15-16 days old, H<sub>37</sub> Ra, 28 days old, *M-lacticola* and *M-phlei*, 4-5 days old and *M-smegmatis*, one week old, were filtered, washed free of media with distilled water and the wet mass of cells pressed against pads of filter paper. Weighed quantities of cells were ground with sufficient Tris buffer (pH 8.6, 0.1 M) to make a fine paste and then made up to give a cell density of 200 mg/ml. This suspension was disrupted in Raytheon sonic disintegrator at 10 kc for 30-40 mins at maximum amperage. The sonicate was centrifuged at 13,000 xg. for 1 hr in a Servall superspeed centrifuge and the clear filtrate used. All operations were carried out at 4-8°. The crude extract was stored in deep-freeze till required.

*Assay of enzyme activity:* Protein was estimated by the biuret reaction and by the 260/280 ratio method of Warburg and Christian<sup>11</sup> when the solution contained smaller amount of protein per ml.

The assay of alanine dehydrogenase was initially carried out by following the pyruvate formation according to the method of Friedmann<sup>9</sup> in which the 2-4-dinitrophenylhydrazone of pyruvate was estimated. When the enzyme was found to be DPN-specific, the assay was also carried out spectrophotometrically. The assay mixture for oxidative deamination reaction contained in micromoles, Tris buffer, pH 9.8 (50), *L*-alanine (10), DPN (1) and enzyme protein (100-200 µg). The final volume was 3 ml and the reaction was carried out at 22-25°. The reduction of DPN was followed by absorption measurements at 340 mµ (Beckmann spectrophotometer, DU). The reverse reaction, namely, the reductive amination of pyruvate, is under investigation.

One unit of alanine dehydrogenase is defined as that amount of enzyme which catalyses the rise in O.D. of 0.01 per minute, at 340 mµ, taking the rate of oxidation at 22-25° for first 2 minutes and specific activity as units per mg of protein, under the above conditions.

## Results

*Purification of alanine dehydrogenase:* Though crude extracts of all the strains used showed alanine dehydrogenase activity, purification was attempted only on the extract of *M-tuberculosis* H<sub>37</sub> RV and that of *M-lacticola*. Organic solvents like alcohol and acetone were found to be unsuitable for purification of the enzyme as they inactivated the protein. The purification steps with A. R. ammonium sulphate and the characteristics of the enzyme are briefly given below.

All steps were carried out at 2-5°. A small sample of the respective fractions was taken for assay of enzyme activity, while the bulk of the fractions was kept in deep freeze till required for purification.

1. *Precipitation with manganous sulphate:* To the crude extract was added 1 M solution of MnSO<sub>4</sub> in water up to 20 per cent of the volume with constant stirring to remove nucleic acid. After keeping for 30 mins, the precipitate formed was removed by centrifuging. The supernatant was dialysed for 24 hrs against Tris buffer, 0.01M pH 7.6.

2. *First ammonium sulphate fractionation:* The dialysed supernatant from above step was fractionated at pH 7.6 with solid (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. The fraction precipitating up to the saturation limit 0.4 was discarded and the fraction obtained between 0.4-0.8 saturation was centrifuged at 13,000 xg for ½ hr. This precipitate was dissolved in Tris buffer pH 8.6, 0.1 M and dialysed against Tris buffer pH 8.6, M/50, for 24 hrs.



3. *Second ammonium sulphate fractionation:* The dialysed solution from previous step was again fractionated with alkaline  $(\text{NH}_4)_2\text{SO}_4$ , prepared by adding 5 ml of conc.  $\text{NH}_4\text{OH}$  to 100 ml of saturated solution of  $(\text{NH}_4)_2\text{SO}_4$ . The fraction obtained at 0.35 saturation, kept for 2 hrs was centrifuged at 5,000 xg for 30 min. and discarded. The supernatant was brought to a saturation of 0.60 by addition of sufficient quantity of alkaline ammonium sulphate and kept for 4 hr. The fraction obtained was centrifuged at 13,000 xg for  $\frac{1}{2}$  hr and retained. This was dissolved in known volume of 0.1 M Tris buffer, pH 8.6 and dialysed against M/50 Tris buffer, pH 8.6 for 24 hr.

4. *Gel adsorption and elution:* A known volume of the enzyme solution whose pH was adjusted to 5.2, was mixed with calcium phosphate gel prepared by the method of Kiellin and Hartree<sup>12</sup> to obtain a protein-gel ratio of 2. The gel was centrifuged and removed. The supernatant which contained alanine dehydrogenase was now adjusted to pH 8.6 and fresh gel was added to give again the same ratio, stirred for 5 min. and centrifuged out. This gel was saved and enzyme extracted with phosphate buffer of pH 8.6. The active eluant was centrifuged and the supernatant used.

*Characteristics of the enzyme:* The enzyme was stored at  $-10^\circ\text{C}$  in 0.1 M Tris buffer pH 8.6. Under this condition the enzyme is stable for several weeks.

The maximum activity of the enzyme was obtained at pH 10, when tested in Tris and glycine buffers over the range 6 to 11 using 50  $\mu$  mole of buffer constituents (Fig. 1). The

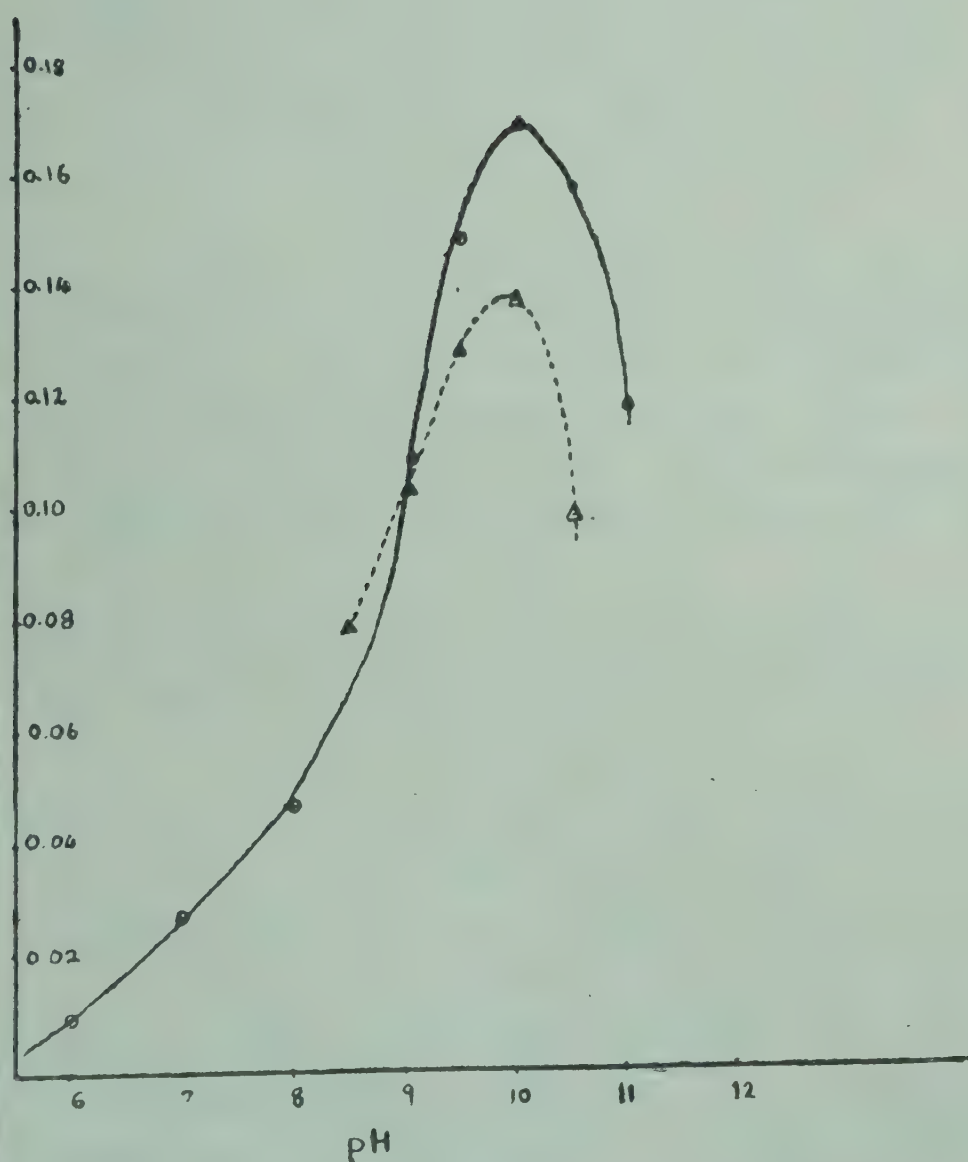


FIG. 1. Activity of alanine dehydrogenase against pH of buffer  
 —●— Tris buffer                      Δ-----Δ Glycine buffer  
 Reaction mixture contained 2 ml buffer, 10  $\mu$  moles *L*-alanine  
 10  $\mu$  mole DPN. enzyme protein 40  $\mu$  g made up to 3 ml.

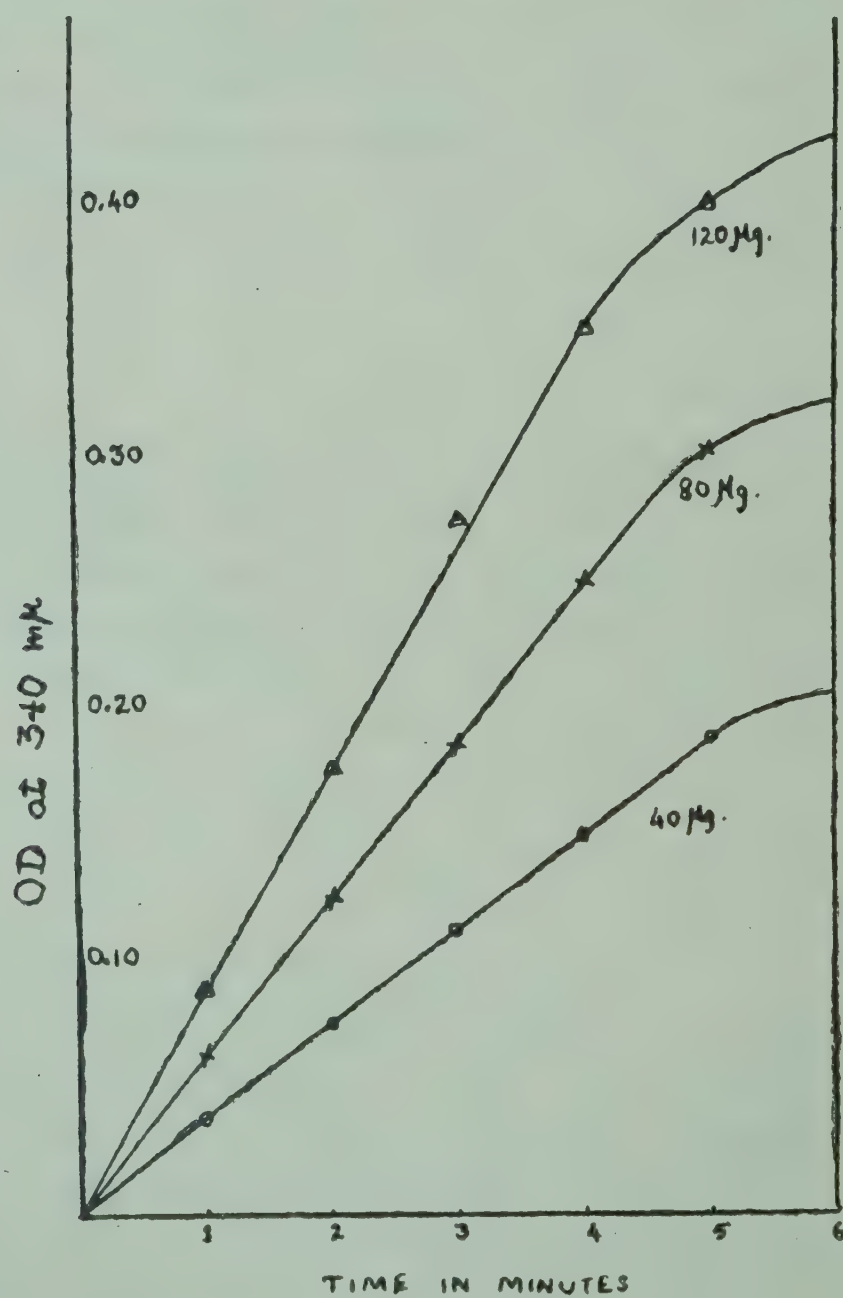


FIG. 2. Activity of alanine dehydrogenase  $\times$  Enzyme conc. Reaction mixture contained in 3 ml: *L*-alanine 10  $\mu$  moles, DPN 1  $\mu$  mole, Tris buffer 50  $\mu$  moles and enzyme protein as shown above.



effect of different concentrations of *L*-alanine, DPN and enzyme is given in Figs. 2, 3 and 4. The rate of oxidative deamination is linear with respect to time and enzyme concentration up to 4 mins. The Michaelis constant of the enzyme for *L*-alanine was found to be  $1.5 \times 10^{-3}M$ .

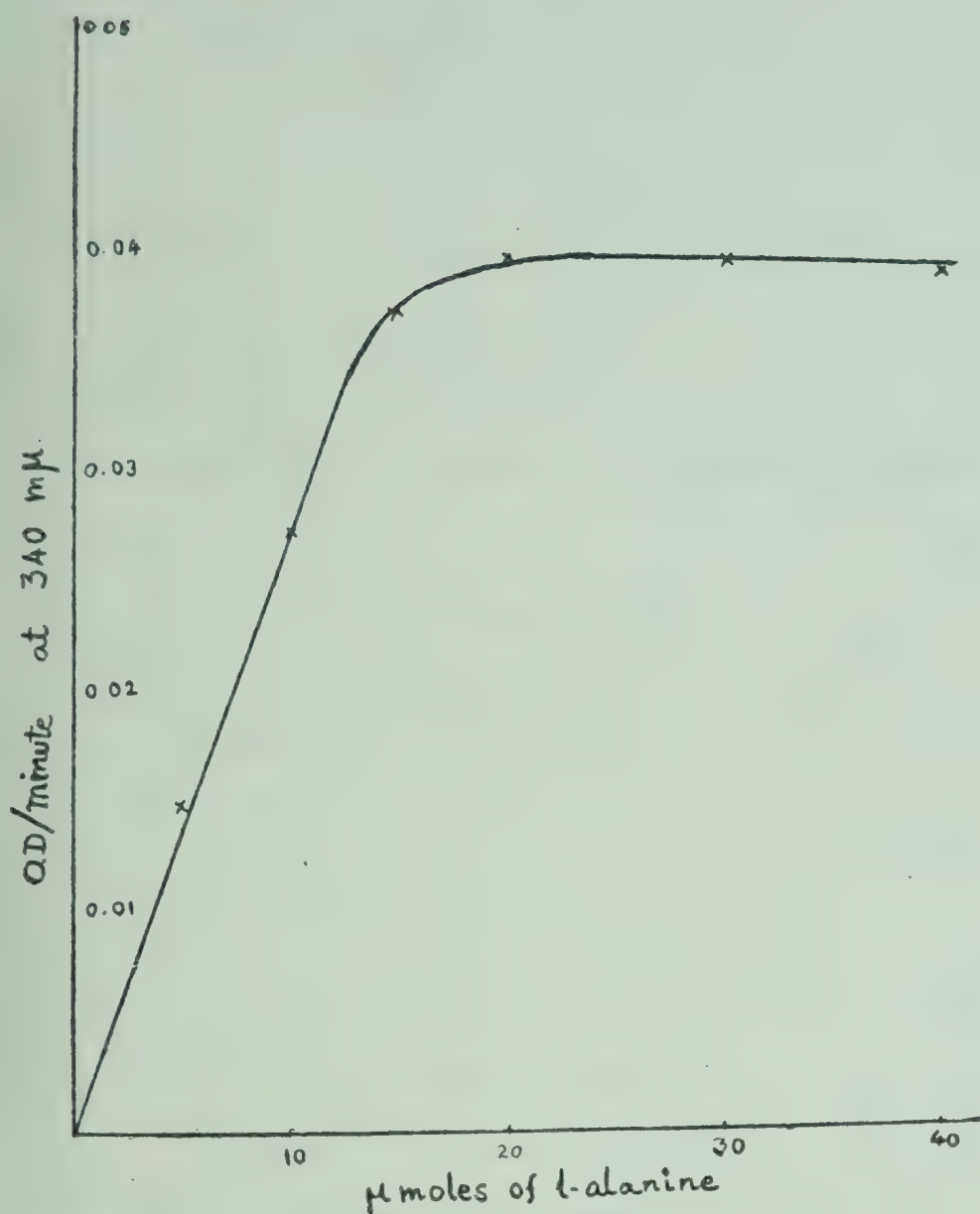


FIG. 3. Alanine dehydrogenase activity  $\times$  conc. of *L*-alanine. Reaction mixture: 40  $\mu$ g of enzyme protein, 1  $\mu$  mole of DPN, 50  $\mu$  moles of Tris buffer and different concentrations of *L*-alanine. Total Volume=3 ml.

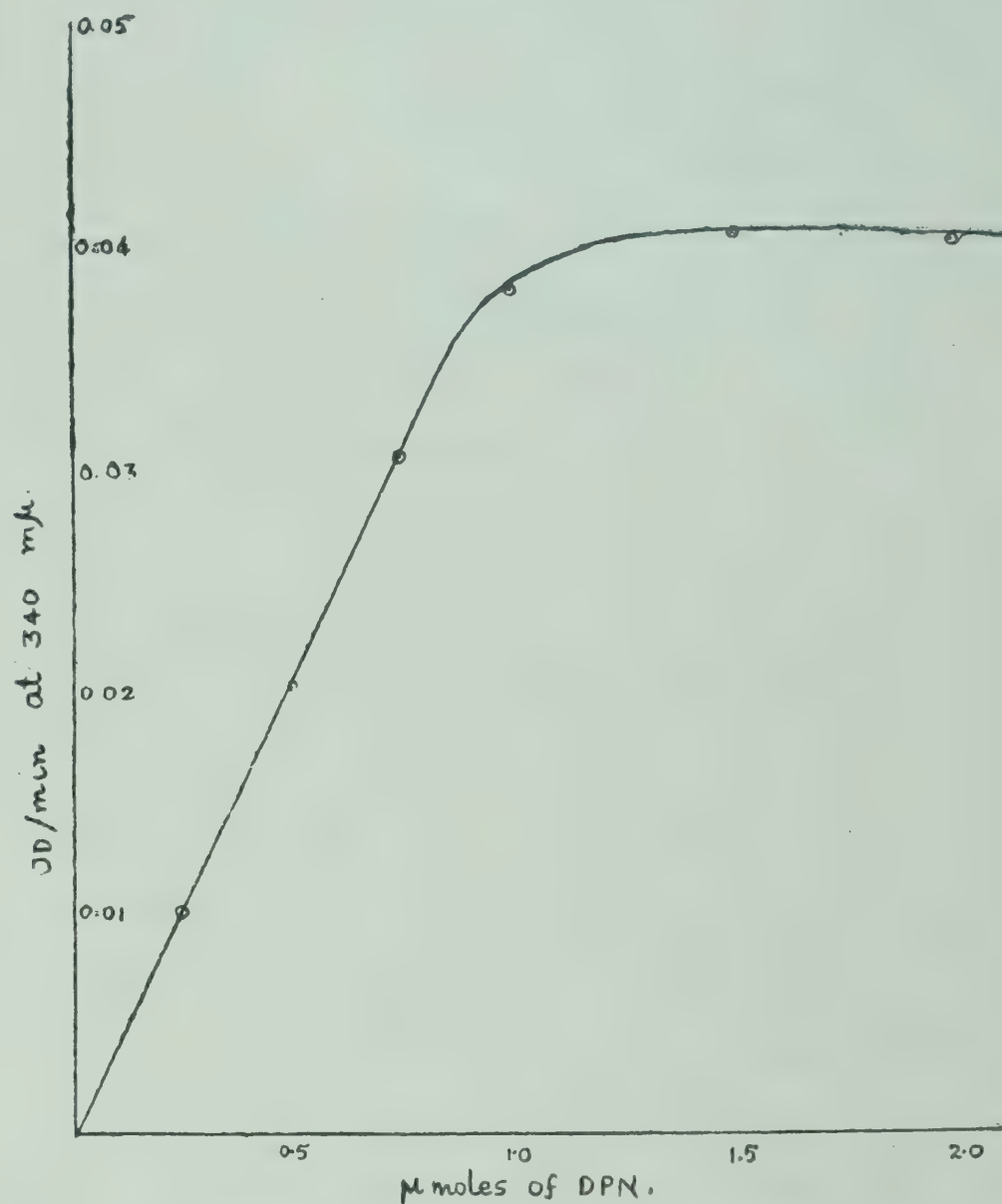


FIG. 4. Alanine dehydrogenase activity  $\times$  different conc. of DPN. Reaction mixture contained in 3 ml: 40  $\mu$ g enzyme protein, 10  $\mu$  moles *L*-alanine, 50  $\mu$  moles Tris buffer and different concentrations of DPN

The enzyme is soluble in alkaline pH and is fairly stable at room temperature in crude extract. But the purified preparation loses activity fairly rapidly.

Iodoacetic acid and PCMB inhibited the enzyme considerably showing the participation of sulfhydryl groups. There was appreciable stimulation of the enzyme by 8-hydroxyquinoline, INH and EDTA in the initial stages (Table III).

The enzyme showed absolute specificity for *L*-alanine as substrate. Other amino-acids tried were not utilised as substrate.

The pyruvate formed was identified chromatographically on filter paper with 2 solvent systems using 2-4-dinitrophenylhydrazine as the detecting reagent. A single spot was shown in both solvents.

The results of purification of the enzyme are shown in Tables I and II.

## Discussion

The results show that the enzyme preparation obtained above specifically catalyses the oxidative deamination of *L*-alanine to pyruvate with DPN as the co-factor. FAD



TABLE I  
*Purification of alanine dehydrogenase from M-lacticola*

Fraction	Total vol. ml	Protein × mg/ml	Total protein mg	Specific activity	Total activity	Purification
1. Crude extract ...	30	8.0	240	1.89	359.6	...
2. MnSO <sub>4</sub> precipitation ...	40	2.4	96	3.10	297.6	1.5 ×
3. First (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> fraction (40-80)	20	1.80	36	6.10	219.6	3.2 ×
4. Second (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> fraction (40-60)	20	0.70	14	12.0	168.4	6.3 ×
5. Calcium phosphate gel eluate ...	20	0.25	5	20.6	102.9	10.1 ×

TABLE II  
*Purification of alanine dehydrogenase from M-tuberculosis H<sub>37</sub> RV.*

Fraction	Total vol. ml	Protein × mg/ml	Total protein mg	Specific activity	Total activity	Purification
1. Crude extract ...	10	5.2	52	1.20	53.04	...
2. MnSO <sub>4</sub> precipitation ...	14	2.3	32.2	4.01	129.12	3.3 ×
3. First (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> fraction (40-80) ...	10	1.2	12	9.84	121.28	8.2 ×
4. Second (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> fraction (40-60)	10	0.52	5.2	17.16	89.23	14.3 ×
5. Calcium phosphate gel eluate ...	5	0.21	1.05	37.34	39.14	31.2 ×

TABLE III  
*Effects of metal chelates and inhibitors on alanine dehydrogenase*

Substance used	Final concentration	Activity of the enzyme
None ...	...	100
Arsenite ...	1 × 10 <sup>-5</sup> M	105
PCMB ...	1 × 10 <sup>-5</sup> M	00
Iodoacetic acid ...	1 × 10 <sup>-3</sup> M	40.6
8-hydroxyquinoline ...	1 × 10 <sup>-5</sup> M	121
EDTA ...	1.6 × 10 <sup>-3</sup> M	140
INH ...	1 × 10 <sup>-3</sup> M	128

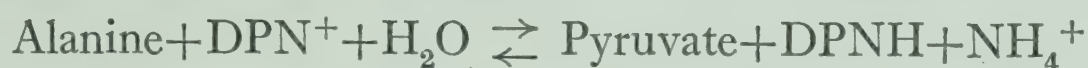
INH: Isonicotinic acid hydrazide.  
EDTA: Ethylenediamine tetraacetic acid.  
PCMB: Parachloromercurybenzoate.



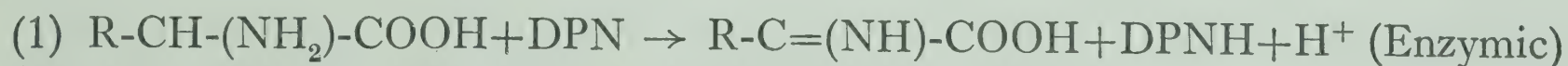
has no activating effect on the enzyme which shows that the amino acid oxidase is absent. TPN also does not activate the enzyme. The optimum pH is 10 and at neutral pH, the activity is very low. The enzyme is soluble in alkaline pH and is fairly stable at room temperature. Crude enzyme kept in deep-freeze remains active at least for a month, while purified enzyme loses some activity. The most stable pH for the enzyme is 8.6.

The absolute specificity of substrate indicates a definite role for the enzyme at least in *Mycobacteria*. The presence of the enzyme in all strains used suggests a possible role in the synthesis of *L*-alanine from pyruvate and  $\text{NH}_3$ . Ammonia is incorporated in the medium as  $(\text{NH}_4)_2\text{SO}_4$  while pyruvate could be available from glycerol utilization. The reductive amination of pyruvate with  $\text{NH}_3$  and DPNH has, however, not been tried, although in all other properties the enzyme closely resembles the alanine dehydrogenase purified and studied by Goldman<sup>3</sup>. Among the amino acids tried, namely, *L*-alanine, glycine, aspartic acid, glutamic acid, serine, cysteine, valine,  $\alpha$ -aminobutyric acid,  $\beta$ -alanine and phenylalanine, only *L*-alanine serves as the substrate. D-alanine is neither a substrate nor an inhibitor. This suggests that the enzyme may be used for the assay of *L*-alanine or its detection as impurity in D-alanine preparations. The high inhibition shown by PCMB and iodoacetic acid suggests the active participation of sulphhydryl groups. Arsenite did not show any inhibition. 8-Hydroxy-quinoline and EDTA showed appreciable initial activation of the enzyme, suggesting that the removal of metallic ions which probably oxidise sulphhydryl groups promotes enzyme activity. INH also showed initial activation of the enzyme.

The absence of glutamic dehydrogenase even in catalytic amounts and the absence of pyruvic dehydrogenase in purified preparation indicates that the reaction catalysed by alanine dehydrogenase is obviously the one proposed by Goldman<sup>3</sup>.

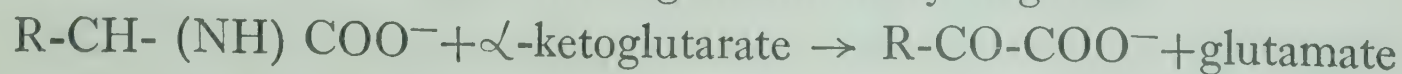


The mechanism proposed by von Euler<sup>1</sup> for the oxidative deamination of an amino acid involves two steps. In the first step, the amino acid reacts enzymatically with DPN to give an imino compound which in the second step gets hydrolysed non-enzymatically to give the keto acid and ammonia.



Strecker<sup>13</sup>, from an analysis of kinetic data for glutamic dehydrogenase, and Goldman<sup>3</sup>, from his studies on alanine dehydrogenase, refute this mechanism. No attempt has been made to study the mechanism or to detect the iminopropionate. However, the fact that the purified alanine dehydrogenase is active at only high pH suggests that the imino-compound might in fact be an intermediate. In the formation of this compound  $\text{H}^+$  ions are released and therefore addition of  $\text{H}^+$  ion, i.e., a low pH, will force the reaction backwards to form alanine. A high pH, on the other hand, will direct the reaction towards the disappearance of alanine.

The absence of glutamic dehydrogenase rules out the possibility of a linked system of reaction as proposed by Braunstein and Asarkh<sup>5</sup> and Nisman<sup>6</sup> which links a glutamic- $\alpha$ -ketoacid transaminase with a glutamic dehydrogenase:





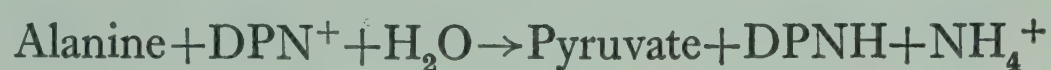
In our preparation the absolute absence of DPN reduction in the presence of 200  $\mu$ g of enzyme protein with 20  $\mu$  mole of glutamic acid rules out this possibility.

It is implicit that we assume a basic similarity of mechanism of action between glutamic and alanine dehydrogenases. A kinetic analysis for alanine dehydrogenase has been done by Goldman<sup>3</sup>. The enzyme kinetics for reductive amination of pyruvate with DPNH and  $\text{NH}_3$  in our preparation is in progress. The enzyme has been so far shown to be present only in microorganisms and not in any animal or plant tissue. As such, it is too early to speculate upon the role of such an enzyme from the point of view of comparative biochemistry.

Detailed studies on the enzyme kinetics, mechanism of action, action of anti-tubercular drugs, and reversal of inhibition by metal chelates and sulphydryl activating compounds are under study.

## Summary

Alanine dehydrogenase activity has been shown in the cell free extracts of a few strains of Mycobacteria. The purified enzyme from *M-lacticola* and *M-tuberculosis* H<sub>37</sub> RV, has been shown to be specific for *L*-alanine and catalyses the reaction.



The characteristics of the purified enzyme are given and its possible role in metabolism discussed.

## Acknowledgment

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*Discussion*

Q. How would you interpret the activating effect of metal-sequestering agents?

A. They may remove some heavy metals which have combined with SH-groups and thus help in regenerating these active groups.

Q. Were any ions found to exert an activating effect?

A. No.

Q. Were the  $K_m$  values for the two substrates different?

A. They were not significantly different.



# MICROBIOLOGICAL EVALUATION OF HEART EXTRACTS

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Although heart extracts are employed as useful remedies against cardiovascular diseases, suitable methods for assaying their therapeutic potency or even controlling their quality have not been worked out so far. In view of the fruitful results obtained earlier in the standardization of certain therapeutics<sup>1-3</sup>, a microbiological approach was made towards the evaluation of heart proteins and heart extracts and the results recorded are presented in this paper. It is of interest to mention here that *Mycobacterium carotinogen*<sup>4</sup>, subsequently identified as *Corynebacterium carotenogenum*,<sup>5</sup> has successfully been employed for determining the potency of liver extracts<sup>6-8</sup> as well as in the evaluation of proteins in the heart extracts<sup>9</sup> for the reason that the major constituents of heart extracts proved to be nitrogenous in nature.

## Experimental Materials and Methods

As a first step, amino acid composition of both heart and muscle tissues was determined chromatographically to find out if they differ in their protein composition. The material used for this purpose was derived from goats immediately after slaughtering. The beef heart and the human heart have been the subjects of analysis elsewhere<sup>10,11</sup>.

TABLE I  
Amino acid composition of heart, meat and heart extracts (Goat)

	Goat heart	Goat meat	'Herzolan'	Lab. sample of heart extract
Nitrogen value ...	16.00	16.00	1.75	1.568
Protein value ...	100	100	10.93	9.8
Cystine ...	3.88	2.014	0.27	0.205
Lysine ...	9.9	10.02	1.1	1.0
Histidine ...	3.4	4.5	0.86	0.8
Arginine ...	9.9	9.4	1.06	0.5
Aspartic acid ...	5.1	4.8	0.56	0.31
Serine ...	3.61	3.64	0.33	0.16
Glycine ...	5.8	4.7	0.6	0.34
Glutamic acid ...	14.9	12.91	1.5	1.57
Threonine ...	3.22	3.6	0.76	0.55
Alanine ...	9.1	9.3	0.9	1.0
Tyrosine ...	3.64	3.57	0.156	0.15
Methionine ...	3.6	2.8	0.22	0.04
Valine ...	4.0	3.8	0.08	0.25
Phenylalanine ...	2.5	1.6	0.27	0.2
Isoleucine ...	5.5	5.0	0.45	0.4
Leucine ...	6.5	5.5	0.4	0.4
Tryptophane and Proline ...	Not determined			

Inasmuch as goat hearts are exploited commercially for the manufacture of heart extracts, a sample of heart extract (following the commercial method of preparation) was prepared in the laboratory and was included in this analysis along with a commercial sample of heart extract designated 'Herzolan'. The results are presented in Table I. The

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electrophoretic patterns of the heart and muscle proteins were also followed in the Kerns' apparatus and by adoption of agar electrophoresis<sup>12</sup>, but the results recorded<sup>8</sup> are not reproduced here as they do not contribute to the present discussion.

For determining the differences, if any, in the microbiological responses to the proteins and/or growth factors of the heart and heart extracts, the following cultures were employed: (1) *Streptococcus viridans* NCTC 3166, (2) *Streptococcus fecalis* R, (3) *Clostridium botulinum* NCTC 7272, (4) *Clostridium septicum* NCTC 548, (5) *Clostridium chauvoei* NCTC 287, (6) *Leuconostoc mesenteroides* ACTC 8042 and (7) *Lactobacillus arabinosus* 17-5.

The choice of *S. fecalis* needs no special mention here as it had been used successfully in determining the protein values of the heart extracts previously<sup>9</sup>. Preference was given to *S. viridans* over *S. pyogenes* for reason of the former's affinity to the heart tissues as evidenced by its causation of carditis. The use of 'cooked-meat medium' for the cultivation of the pathogenic clostridia suggested their employment in the present study and the choice of the species was restricted only by their non-availability readily. *Leuconostoc* and *lactobacillus* species are recognized for their demands for amino acids and growth factors and their inclusion, therefore, was considered worthwhile for indicating the availability or otherwise of all the amino acids and growth factors demanded by them for their growth. *Corynebacterium carotenogenum* was excluded from this study as its response to these materials has been the subject of a detailed investigation before.

The maintenance medium for clostridia was Robertson's cooked meat medium. The streptococci species were maintained on meat infusion peptone agar and the rest were cultured on simple nutrient agar. Saline suspensions of the cultures (two drops of a thin suspension for each tube) were used as inoculum.

For microbiological evaluation, both infusions and enzyme digests were made use of and they were, in brief, prepared the following way.

(i) *Heart infusion*. This was made from fresh goat heart. The heart muscle was freed from fat and connective tissues and was minced. For every 50 g portion, 100 ml distilled water was added and allowed to extract at 4°C for 24 hours. It was filtered through muslin, residue pressed out and the combined filtrate refiltered through a filter paper, steamed for 1 hour and again filtered. The extracts usually averaged 105 ml and were equivalent to about 0.47 g of fresh heart per ml. The nitrogen content of this preparation was about 0.089 per cent.

(ii) *Meat infusion*. This was prepared in the same way as heart infusion using muscle tissues (red) instead of heart tissues. The final infusion obtained was about 106 ml equivalent to, again, 0.47 g of the tissue but contained more nitrogen, viz., 0.149 per cent.

(iii) *Enzymatic digest of heart*. 50 g minced heart were mixed with 100 ml of 0.5 per cent pepsin solution and 1 ml of HCl to bring the pH to 5.0 and the mixture incubated at 37°C for 48 hours after incorporation of some toluene. Then the mixture was adjusted to pH 8.4 with NaOH, buffered with borate+KCl solution of pH 8.0 and digested with 300 mg of pancreatin and 100 mg trypsin (1.25 U.S.P.) for a further period of 72 hours at the body temperature. The mixture was filtered, and after readjusting the pH to 7.0, steam-heated for 10 minutes, cooled and again filtered. The nitrogen value of the final preparation was 0.38 per cent (0.312 g of fresh heart per ml).

(iv) *Enzymatic digest of muscle*. This was prepared in the same way as (iii) and gave a nitrogen value of 0.46 per cent (0.262 g of fresh meat per ml).



The four substrates were then examined for their suitability for the growth of test bacteria after adjusting their nitrogen contents at various levels (indicated in Table II) in a medium which contained only 1 per cent glucose and 0.5 per cent NaCl. Rest of the procedure followed was similar to any microbiological assay and the growth responses were measured in terms of turbidity in a Hilger Colorimeter using filter No. 8 (675 mμ) and 0.25 cm cells. All the culture tubes were heated at 100°C in a water bath for 15 minutes (to kill the pathogens) before the readings were made and recorded in terms of 'Absorbancy'. The results are presented in Table II excluding those of *C. chauvoei* which failed to grow in all the media.

Since *C. septicum* proved to be more sensitive to heart extracts than the rest of the bacteria put to the test, an attempt was made to see if this bacterium could be of any use for assessing the potency of commercial heart extracts. The results recorded with some of the samples are shown in Table III. To indicate to what extent the heart extracts are superior to other preparations mentioned before, the response elicited with *C. septicum* is illustrated in Fig. 1.

Results and Discussion

From the results given in Table I, it is clear that no significant differences exist between the amino acid composition of the heart and the muscle proteins. Supporting evidence for this finding was derived from the electrophoretic patterns obtained for these proteins,

TABLE II  
Growth response of the test bacteria to heart and meat infusions and digests  
(Hilger readings : Absorbancy)

Nitrogen source in mg/ml	Test bacteria					
	S. viridans	S. fecalis	C. septicum	C. botulinum	L. arabinosus	L. mesente- roides
Heart infusions: Nil ...	1.0	1.0	1.0	1.0	1.0	1.0
20 mg ...	0.906	0.925	0.773	0.983	0.816	0.966
30 mg ...	0.810	0.921	0.627	0.979	0.699	0.931
40 mg ...	0.773	0.903	0.569	0.975	0.638	9.911
50 mg ...	0.710	0.883	0.511	0.975	0.531	0.893
Heart digest: Nil	1.0	1.0	1.0	1.0	1.0	1.0
20 mg ...	0.996	0.950	0.935	0.992	0.909	0.987
30 mg ...	0.971	0.939	0.929	0.987	0.842	0.971
40 mg ...	0.971	0.921	0.908	0.987	0.822	0.968
50 mg ...	0.983	0.903	0.906	0.987	0.799	0.962
Meat infusion: Nil	1.0	1.0	1.0	1.0	1.0	1.0
20 mg ...	0.950	0.943	0.903	0.992	0.929	0.979
30 mg ...	0.924	0.931	0.864	0.987	0.867	0.962
40 mg ...	0.886	0.917	0.796	0.987	0.810	0.950
50 mg ...	0.858	0.878	0.767	0.987	0.770	0.939
Meat digest: Nil	1.0	1.0	1.0	1.0	1.0	1.0
20 mg ...	0.903	0.947	0.931	0.983	0.889	0.983
30 mg ...	0.864	0.939	0.919	0.983	0.836	0.971
40 mg ...	0.796	0.919	0.903	0.979	0.816	0.958
50 mg ...	0.761	0.900	0.883	0.979	0.786	0.954



TABLE III  
*Growth response of C. septicum to heart infusions*  
 (Concentration of Herzolan in 10ml basal medium was 0.5 ml)

Heart infusion		Hilger reading absorbancy
Fresh heart	...	0.943
Laboratory made	...	0.958
Commercial: B. No. 1517	...	0.983
" " 72/CB	...	0.958
" " 77/CB	...	0.979
" " 80/CB	...	0.962
" " 84/CB	...	0.979
Uninoculated (Control)	...	1.0

Growth response of *C. septicum* to heart and meat extracts

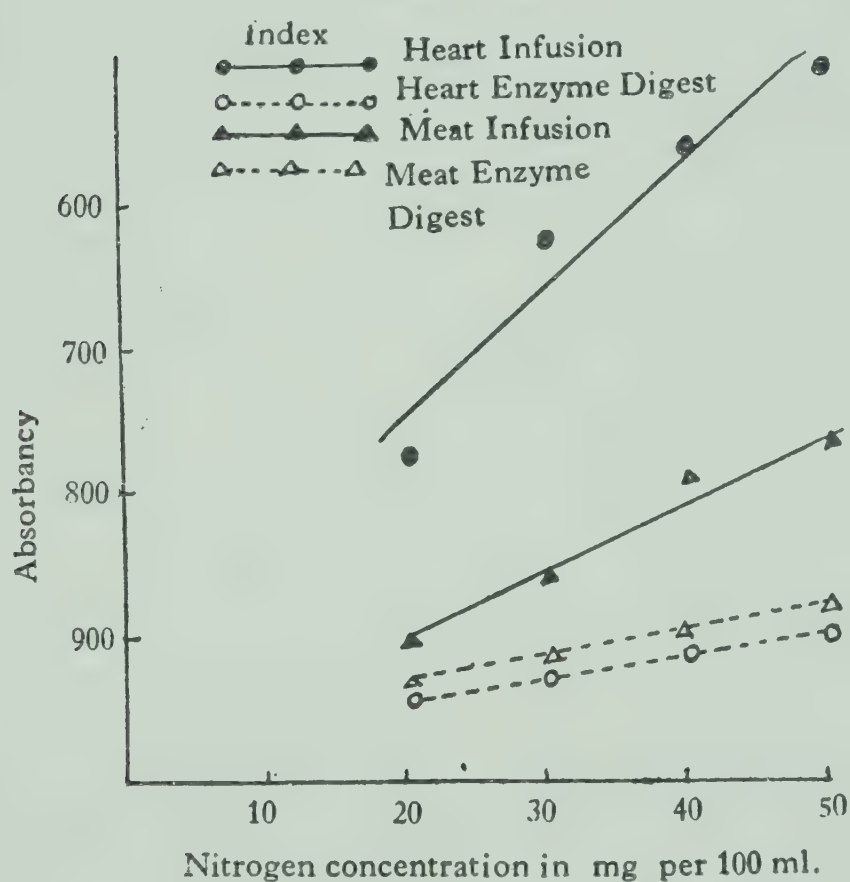


FIG. 1

which also did not reveal any differences whatsoever. The amino acid values recorded for the heart extracts prepared in the laboratory and those achieved for the commercial preparation 'Herzolan' also agreed well within the experimental errors, suggesting thereby that about 10 per cent of the proteins of the organ can invariably be recovered in its extracts made for therapeutic purposes. The protein values calculated in the present instance (from the nitrogen values), in fact, have been obtained experimentally<sup>9</sup> by using both *Streptococcus fecalis* R and *Corynebacterium carotenogenum*, thus lending support to the view that microbiological approach may, with advantage, be adopted in the quality control of these preparations until a suitable procedure is worked out for determining their potency.



Results shown in Table II indicate that both the infusions and the digests can meet adequately the nutritional demands of six of the seven bacteria including even the most fastidious species. It is of interest that heart infusion and meat digests, unlike heart digest and meat infusion, are favourable to the growth of *S. viridans* and that heart infusion in particular boosts its growth to a considerable degree. In fact, the response shown by this bacterium in the heart infusion is in striking contrast with that elicited by *S. fecalis* and offers an explanation for the affinity of the organism to heart tissue. It is also evident that infusions are generally more favourable for the growth of the bacteria than are the respective enzyme digests, a finding which fully supports the age-old usage of extracts (infusions) for the preparation of bacteriological media in preference to all other types of preparations. The general observation that the growth response was proportional to the nitrogen levels (maintained by the addition of the various preparations) suggests that the factor promoting the growth is nitrogenous, presumably proteinic. A more critical examination of the results indicates that heart infusions are more favourable for growth than are the meat preparations and that the response elicited by *C. septicum* to the former was particularly of a sensitive nature (Fig. 1) to suggest its use for the quality control of heart extracts. The exact nature of the substance associated with the heart extracts and responsible for promoting the growth of the *Clostridium* species has not been elucidated so far. It is hoped that further investigations on the goat and ox preparations will throw light on this interesting problem.

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*Discussion*

- Q. The therapeutic use of heart extracts have become outmoded with the advent of more powerful cardiovascular drugs. What relation has the microbial evaluation to the expected pharmacological action?
- A. The pharmacological action of the extracts evidently depends on the proteins and other constituents extracted from the heart muscle which also promote bacterial growth. Hence the latter could be used as a measure of the active components.
- Q. Has any correlation between vasodilator or other pharmacological property of the extracts and their microbial-growth promoting activity been established?
- A. Such correlative studies will be carried out before recommending the procedure for routine evaluation of heart extracts.



# THE ANTIGENICITY OF HUMAN SPERMATOZOA AND ITS SIGNIFICANCE

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The antigenicity of bull spermatozoa was first demonstrated by Landsteiner<sup>1</sup>. This finding has since been confirmed with other mammalian spermatozoa. The presence of specific antibodies in the serum of the animals injected with spermatozoa was usually demonstrated by the specific property of such a serum to agglutinate and immobilize homologous spermatozoa. Weil *et al.*<sup>2</sup> reported the presence of 10 antigens in human seminal plasma. However, a detailed investigation of the antigenic composition of human and buffalo semen was first reported by Rao and Sadri<sup>3,4</sup>. The work reported here concerns the nature of the human sperm antigens and their significance.

## Experimental Materials and Methods

Semen, blood serum and other secretions of the reproductive tract were obtained from men referred for infertility and also from fertile donors. Cervical mucus and also blood serum were obtained from women referred for infertility and also from normal fertile women. Antisera to human semen, cervical mucus and blood serum were obtained from rabbits immunized with the respective antigens.

Chromatographic determination of amino acids was according to the method of Giri and Rao<sup>5</sup> using circular paper chromatography. The method of Consden, Gordon and Martin<sup>6</sup> (1944) was used for two dimensional chromatography.

The presence of auto- and iso-antibodies to spermatozoa in the blood sera of infertile couples was detected by the haemagglutination technique of Boyden as modified by Stavitsky<sup>8</sup>. The details of the technique have already been given<sup>3</sup>.

## Experimental

*Antigens of human spermatozoa:* The work carried out on the antigenic composition of human semen has already been reported<sup>3</sup>. It was observed that human seminal plasma has at least 16 antigens and spermatozoa 7. Seminal plasma was observed to contain 6 antigens in common with blood serum, whereas spermatozoa had only one antigen in common. In the semen samples of 3 men referred for infertility, spermatozoa showed a tendency to agglutinate. Spermatozoa which were free and motile soon after ejaculation started agglutinating later on. The clumps became bigger with increase in time. Agglutination of spermatozoa was suggestive of something in the seminal plasma responsible for such agglutination. In one of the 3 samples, spermatozoa showed clumping even when the semen was examined soon after ejaculation. Usually, agglutination is brought about by specific antibodies and it was of interest to find out whether such antibodies were present in the seminal plasma and blood serum of the individuals



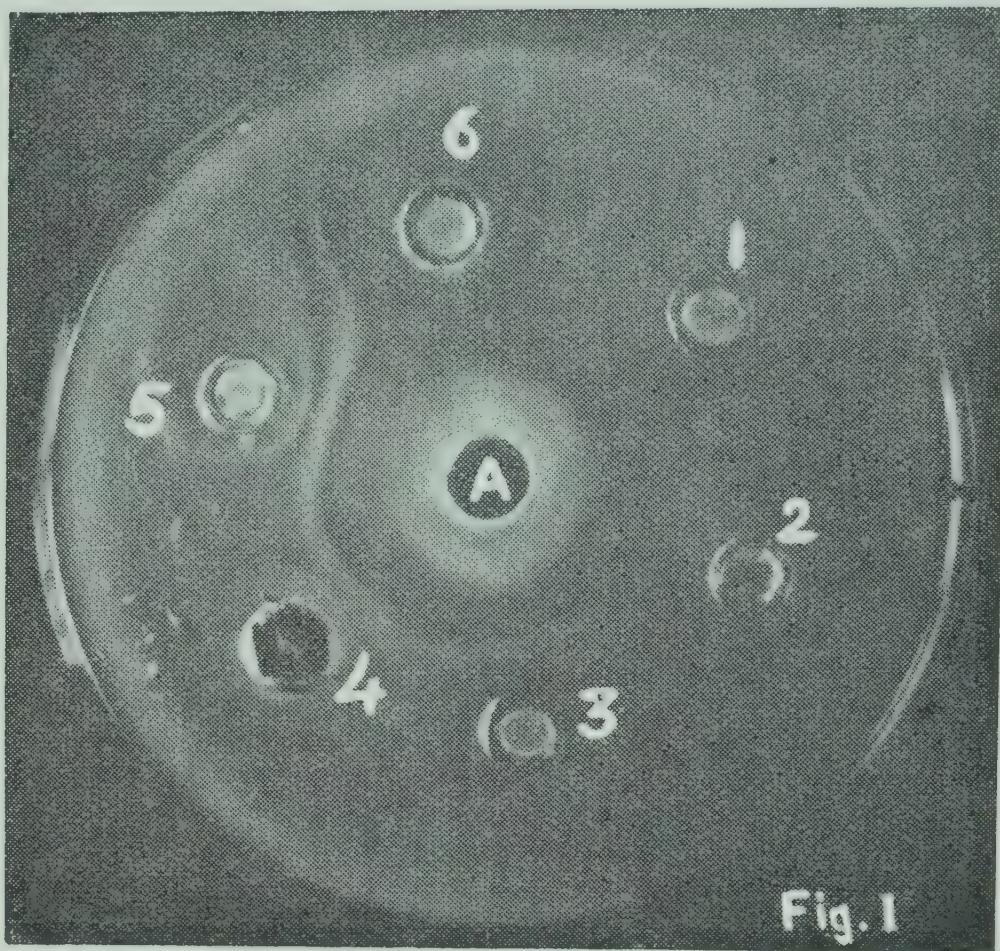
whose semen showed the presence of agglutinated spermatozoa. It has already been shown that blood serum and seminal plasma have common antigens.

In one of the Ouchterlony agar plates, originally set up to find the antigenic composition of human cervical mucus, freeze-thawed semen and blood serum from such an individual were kept side by side. Four days after the antigens and antisera were placed in the agar plates, a precipitin line developed between the blood serum and the freeze-thawed spermatozoa (Fig. 1). The precipitin line indicated that antibodies were present in the blood serum against some of the seminal antigens, for it was unlikely that antibodies to blood proteins would be present in seminal plasma.

FIG. 1

A. Antibody to human cervical mucus.

1. Cervical mucus.
2. Buffalo frozen-thawed semen.
3. Buffalo cervical mucus.
4. Seminal plasma.
5. Frozen-thawed semen.
6. Blood serum.



*Experimental proof that spermatozoa have antigens not common to either seminal plasma or blood serum:* The absorption test was carried out to find out whether human spermatozoa have specific antigens which are not present either in seminal plasma or in blood serum.

Rabbit antiserum to human semen was absorbed with azoospermic semen (semen showing absence of spermatozoa). Azoospermic semen and not seminal plasma was used for the absorption so as to exclude the possibility of any spermatozoal antigens in the fluid used for absorption. It has already been shown<sup>9,10</sup> that enzymes and other constituents of spermatozoa leak into seminal plasma. Equal quantities of azoospermic semen and rabbit antiserum to human freeze-thawed semen were mixed and kept at 37°C for 30 minutes with 1:100 merthiolate as preservative. The absorbed antiserum was placed in the centre of an agar plate and round it were placed seminal plasma (separated from a normal semen sample with spermatozoa) blood serum, whole semen, cervical mucus, freeze-thawed suspension of spermatozoa and unabsorbed antiserum. The unabsorbed antiserum was placed between seminal plasma and spermatozoa. At the end of 10 days, it was seen that there were 16 precipitin lines between seminal plasma and unabsorbed antiserum and also between semen and unabsorbed antiserum (Fig. 2). There were about



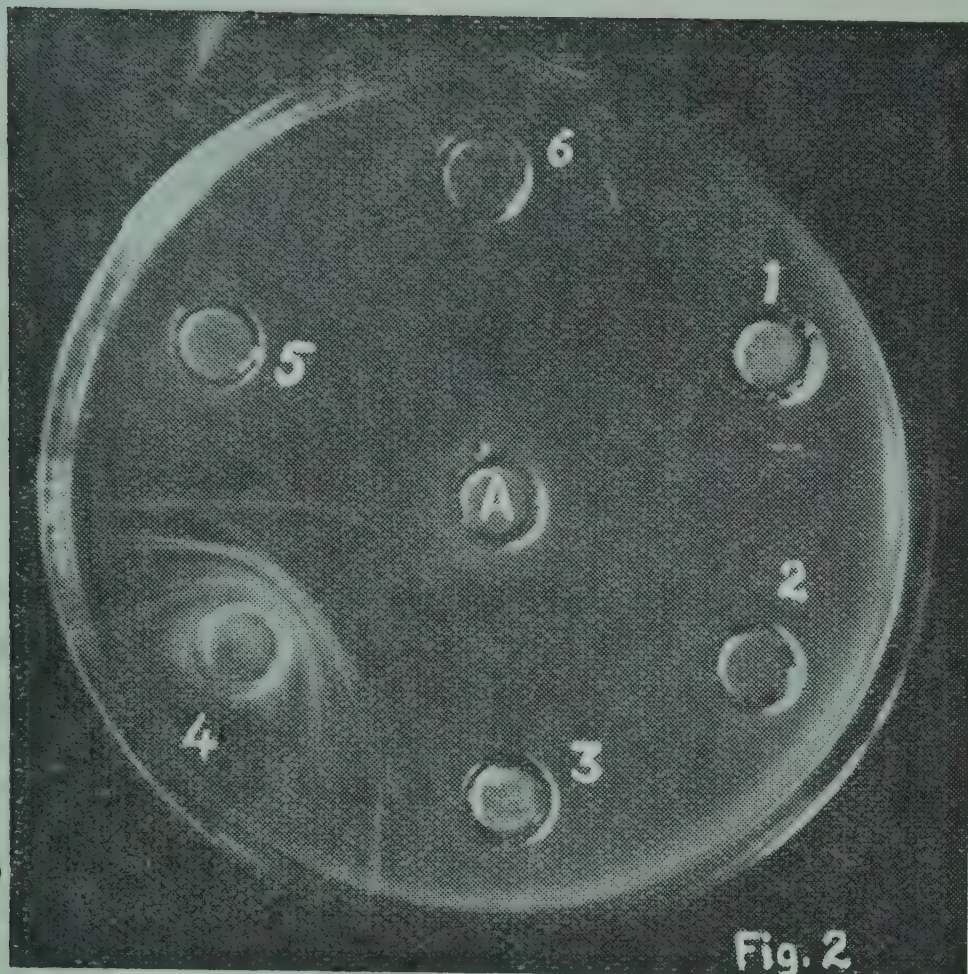


FIG. 2.

A. Antibody to human semen absorbed with azoospermic semen.

1. Frozen-thawed spermatozoa.
2. Cervical mucus.
3. Semen.
4. Antibody to human semen.
5. Seminal plasma.
6. Blood serum.

4 precipitin lines between absorbed and unabsorbed antiserum. This indicated that azoospermic semen used to absorb the rabbit antiserum to human freeze-thawed semen was in excess of that needed to absorb all antibodies present in seminal plasma. Between the freeze-thawed spermatozoa and the absorbed antiserum there were 3 precipitin lines, one of medium intensity and two faint. These precipitin lines indicated that spermatozoa had 3 antigens which are not common to seminal plasma. There were no precipitin lines between blood serum and absorbed antiserum, though with unabsorbed antisemen serum, blood serum always gave at least 6 precipitin lines<sup>3</sup>. The results of the absorption experiments indicated that, of the 7 antigens in spermatozoa, 3 were not common either to seminal plasma or blood serum. The 6 antigens common to blood serum and seminal plasma could be completely absorbed by azoospermic semen.

*Nature of the sperm-specific antigens:* It was of interest to find out whether these antigens are heat labile. In order to test this, a pooled sample of human semen was centrifuged and the seminal plasma was separated. The spermatozoa were washed once with saline and resuspended in saline equal to the volume of plasma removed. Both the seminal plasma and spermatozoal suspension were each divided into five equal portions. One set of tubes containing the seminal plasma and spermatozoal suspension was kept at room temperature. The other sets were heated at 56°, 70°, 85° and 100°C for 30 minutes. The tubes were then cooled to room temperature. The contents of the tubes heated at 85°C and 100°C coagulated. The control samples and those heated were placed in the agar plate against antiserum to human semen. Results are indicated in Table I and Fig. 3.

It was observed that the antigens are inactivated progressively with a rise in temperature. At 56°C, 2 of the seminal plasma antigens and 1 of spermatozoa are inactivated. At 70°C, 10 antigens of seminal plasma and 2 of spermatozoa are affected. At the temperature of 85°C and above, all antigens of both seminal plasma and spermatozoa are inactivated. (Table I).



TABLE I  
Results of the Ouchterlony gel diffusion test with seminal plasma and spermatozoa heated at different temperatures

Temperature	Number of antigen-antibody precipitin lines	
	Seminal plasma	Spermatozoa
Room temperature	16	7
56°C	14	5
70°C	6	2
85°C	Nil	Nil
100°C	Nil	Nil

FIG. 3.

## A. Antibody to human semen.

1. Seminal plasma heated at 70°C.
2. Spermatozoa heated at 70°C.
3. Seminal plasma heated at 56°C.
4. Spermatozoa heated at 56°C.
5. Control spermatozoa.
6. Control seminal plasma.
7. Seminal plasma heated at 85°C.
8. Spermatozoa heated at 85°C.
9. Seminal plasma heated at 100°C.
10. Spermatozoa heated at 100°C.

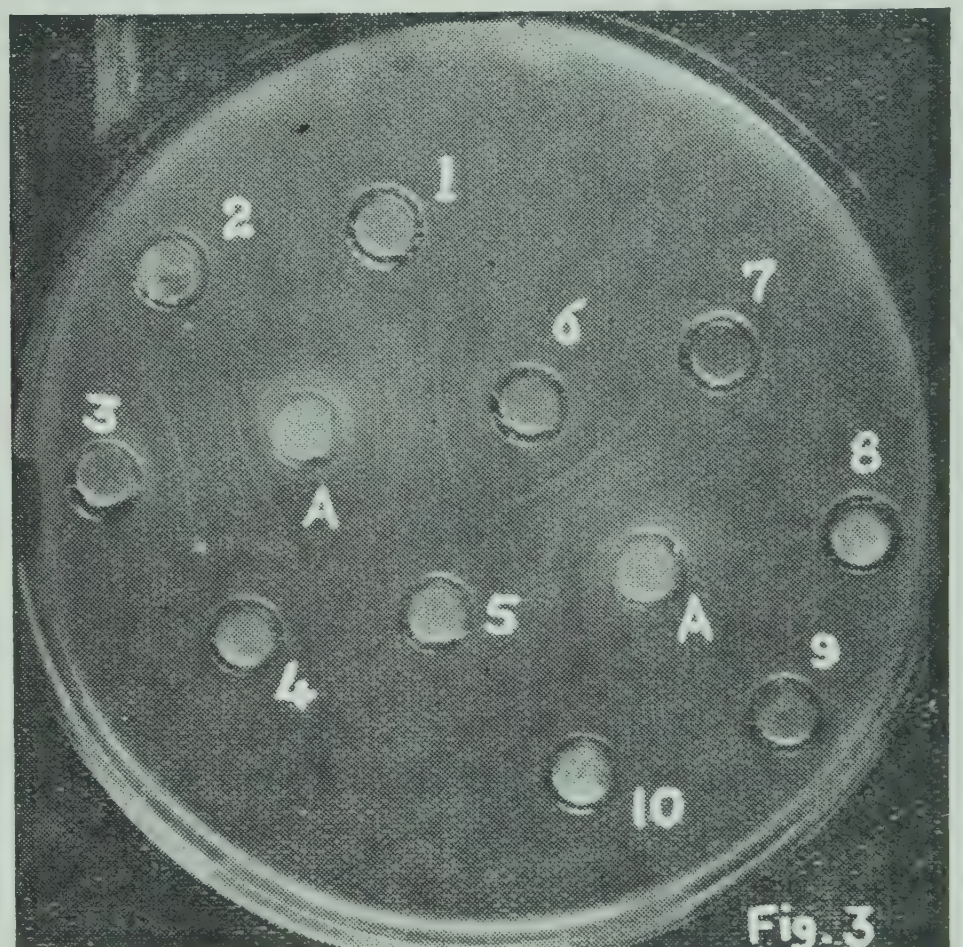


Fig. 3

It was of interest to see whether the antigens of spermatozoa are dialysable and also carry out an amino acid analysis of the freeze-thawed sperm extract. The experiments carried out are detailed below.

Semen samples from normal fertile donors and also from those referred for infertility were pooled. Spermatozoa were separated from seminal plasma by centrifuging at 500 r.p.m. for 30 minutes. They were washed with normal saline and recentrifuged. They were then suspended in water to give a final concentration of 150 million spermatozoa/ml. The suspension was divided into 2 equal parts of 5 ml each. One was kept at 5°C to serve as a control and the other was repeatedly frozen and thawed six times. Both the samples were centrifuged and the supernatants separated. 2.5 ml volumes of both the supernatants were dialysed in a cellophane bag at 5°C for 90 hours, against 6 changes of distilled water. A few drops of toluene were used as a preservative during dialysis. After dialysis, the volume was made up to the original volume of 2.5 ml.



The amino acid analysis of both the dialysed control and the dialysed supernatant of freeze-thawed spermatozoa was carried out using the circular paper chromatography.

Four points equidistant from each other were marked on the circumference of a circle drawn at the centre of a circular Whatman filter-paper of 18.5 cm diameter. 80  $\mu$ l quantities of each of the following were spotted on the points: (1) supernatant of freeze-thawed spermatozoa, (2) supernatant of spermatozoa kept at 5°C, (3) dialysed supernatant of freeze-thawed spermatozoa and (4) dialysed supernatant of spermatozoa kept at 5°C.

Acetic acid: butanol: water, 1:4:5 was used as the developing solvent and the chromatogram was irrigated according to the standard procedure and then allowed to dry and sprayed with ninhydrin reagent. Seven bands developed with  $R_f$ s which suggested the following amino acids.

TABLE II  
*The solutions used for chromatography and the amino acids indicated according to the  $R_f$*

Amino acids detected	Soln 1	Soln 2	Soln 3	Soln 4
1. Lysine ...	+++	+	—	—
2. Aspartic and glutamic ...	+++	++	—	—
3. Serine and glycine ...	++	+	+	+
4. Yellow band (tryptophane) ...	++	+	+	+
5. Tyrosine ...	+	—	—	—
6. Valine ...	++	+	—	—
7. Leucine ...	+++	+	—	—

The identity of these amino acids was determined by the two dimensional chromatography. A known amount of a mixture of standard amino acids as indicated in the table were added to the supernatant of freeze-thawed. The solution was then spotted on a rectangular sheet of Whatman No. 1 filter-paper and developed according to the method of Consden, Gordon, and Martin<sup>6</sup>. Only 9 spots developed. This indicated that there were no more amino acids detected in the freeze-thawed extracts by two dimensional chromatography except those observed in the circular chromatogram. However, the presence of two coloured bands with an  $R_f$  of 0.5 and 0.55 as indicated in the circular chromatogram in the dialysed supernatants was surprising. Evidently these were not due to free amino acids since they could not be removed by dialysis. These may be peptides and will be referred to as peptide 1 and 2. The chromatograms were heated, as it is known that the colour developed by amino acids with ninhydrin fades away on heating. The two spots due to peptide 1 and 2 became more prominent. This property and also the fact they are not dialysable suggests that the bands which react with ninhydrin may be part of a polypeptide.

*Experiments to show the presence of sperm antigens in dialysed spermatozoal extracts:* It was of interest to find out whether the spermatozoal antigens in freeze-thawed extract of spermatozoa dialyse out. Part of the dialysed supernatants, the same as those used for paper chromatography, was used to sensitize sheep erythrocytes in the haemagglutination technique. Care was taken to add enough sodium chloride to the dialysed supernatants to give a final concentration of 0.85 per cent and thus make it isotonic. The erythrocytes



were sensitized with the two supernatants (dialysed and undialysed supernatant of freeze-thawed spermatozoa) and the test carried out using rabbit antiserum to human semen. The antiserum agglutinated the sensitized erythrocytes in both the series giving the same results. 1: 4096 dilution of the antiserum gave a strongly positive (a reaction of<sup>++</sup>) reaction with both sets of sensitized erythrocytes. Thus the results indicated that the spermatozoal antigens present in the freeze-thawed extracts of spermatozoa were not dialysable.

*Use of the spermatozoal antigen to detect iso- and auto-antibodies to spermatozoa:* The sperm specific antigen has been used to detect the presence of small amounts of auto- (antibodies to one's own spermatozoa) and iso-antibodies (antibodies in the wife's blood serum to her husband's spermatozoa) in infertile couples. A survey is underway to find the incidence of such antibodies in the blood serum of infertile couples. The sera of over 350 infertile persons have been analysed. Preliminary results suggest that such antibodies are present in about 4-6 per cent of the infertile couples.

## Discussion

The results of the gel diffusion test carried out have indicated that there are at least 16 antigens in normal human semen. Azoospermic semen and semen from a vasectomized man has already been shown to have only 13 antigens<sup>3</sup>. Spermatozoa have 7 antigens, 4 of which are also common to seminal plasma separated from normal semen as well as to azoospermic semen. Three of the 7 antigens of spermatozoa are specific only to spermatozoa and these are not shared by either seminal plasma or blood serum.

It is possible that seminal plasma and spermatozoa actually have more antigens than indicated by the agar gel diffusion test. This test can only detect the soluble antigens. Also, if two or more precipitin lines are very near each other or overlap, they may give the appearance of a single broad line.

The results of the gel diffusion analysis has shown that seminal plasma have 6 antigens in common with blood serum and are of great interest since it is possible that one of these is a globulin. Antibodies are always associated with globulin fraction of blood serum. It is equally significant that 3 antigens of spermatozoa are not common to seminal plasma or blood serum. On the basis of these findings, it is not surprising to find that antibodies to spermatozoa are present in the blood serum and seminal plasma of certain infertile men. Rao and Sadri<sup>11</sup> reported the presence of diphtheria antitoxin in the seminal plasma of a man immunized with diphtheria toxoid. They have also observed that in the case of a hyperimmunized mare, antitoxin circulating in the blood serum is also present in the cervical mucus. The same can hold true in a woman also since it has been shown<sup>3</sup> that human cervical mucus has at least 3 antigens in common with blood serum.

The survey undertaken to detect auto- and iso-antibodies to human spermatozoa in the blood serum of infertile couples (work carried out in collaboration with Dr A. M. Phadke and G. M. Phadke of Bombay and Dr Edward Tyler of Los Angeles) has indicated that such antibodies when present are likely to prevent conception. Repeated abortions were reported in a couple of women in whose blood serum iso-antibodies were detected. A large number of sera will have to be analysed before a definite role could be assigned to the presence of auto- and iso-antibodies present in the blood serum and certain cases of infertility. The work that is being carried out in collaboration with Dr G. M. Phadke



of the Family Welfare Bureau, Bombay and Dr Edward Tyler of Los Angeles will be reported elsewhere.

Experiments carried out to find the nature of the antigens present in spermatozoa have indicated that they are non-dialysable and protein in nature. It is possible that some of them may get degraded to polypeptides due to proteolysis. The antigens are destroyed by heating above 56°C. Results of circular and two dimensional paper chromatographic analysis of the dialysed and undialysed supernatants from freeze-thawed spermatozoa have indicated the presence of two peptides with  $R_f$  values of 0.5 and 0.55. Since the coloured bands are seen in chromatograms with dialysed extracts also it is likely that these are part of a polypeptide. This assumption is supported by the fact that the colour bands developed due to these two amino acids persist even when the experiments are carried out with dialysed extracts and also these bands do not fade even when the chromatogram is heated. Small quantities of these two are also present in the supernatant from a suspension of spermatozoa stored at 5°C. It has already been shown by Mann<sup>9</sup> and Sheth and Rao<sup>10</sup> that some enzymes and intracellular proteins are released into the medium when spermatozoa are stored at 5°C.

The sperm antigens obtained by repeated freezing and thawing seem to be specific to only spermatozoa. By the use of the haemagglutination technique, these antigens can be conveniently used to detect auto- and iso-immunization to spermatozoa. A preliminary survey carried out has revealed that such antibodies are present in 4-5 per cent of the infertile couples.

### Summary

1. Human spermatozoa have 3 antigens that are not present in either seminal plasma or blood serum.
2. Antibodies circulating in the blood serum are also present in seminal plasma.
3. The antigens of spermatozoa are heat-labile and non-dialysable.
4. The preliminary results of paper chromatographic analysis of the antigens have been reported.
5. It is possible to detect auto- and iso-antibodies to spermatozoa that may be present in the blood serum of some infertile individuals.

### Acknowledgment

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# OCCURRENCE OF FERRIC ION REQUIRING ALKALINE PHOSPHATASE IN PLANTS

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Previous attempts by several workers to demonstrate the occurrence of alkaline phosphatases in plants have not been successful. Giri<sup>1</sup> obtained highly active preparations of phosphatase from Soya bean (*Glycine hispida*). A non-specific phosphomonoesterase was reported by Giri<sup>2</sup> and, later, Ramanarayanan and Giri (unpublished) made a detailed investigation of this enzyme in green gram. Naganna *et al.*<sup>3,4</sup> have reported a  $Mg^{++}$ -activated pyrophosphatase in plants but reported the absence of alkaline  $\beta$ -glycerophosphatase.

In this paper are presented the occurrence of a  $Fe^{+++}$ -requiring alkaline  $\beta$ -glycerophosphatase and some of its general properties.

## Materials and Methods

Sodium  $\beta$ -glycerophosphate was a British Drug House product and the inorganic salts used were of the Analytical Reagent grade. The seeds of green gram used as source of enzyme were procured from the local market.

**Enzyme assay:** The reaction mixtures, unless otherwise stated, contained  $10\ \mu$  moles/ml of veronal-acetate buffer (pH 7.7);  $20\ \mu$  moles/ml of sodium  $\beta$ -glycerophosphate;  $2.9\ \mu$  moles/ml of ferric sulphate; 0.5 ml of enzyme in a total volume of 2.5 ml. After temperature equilibration, the reaction was started by the addition of the enzyme. The tubes were incubated at  $49^\circ$  for 15 min. and the reaction stopped by the addition of 1.0 ml of 20 per cent trichloroacetic acid (TCA). The reaction mixture was centrifuged at 3,000 r.p.m. for 10 min. and 1.0 ml aliquots were used for phosphate assay by the method of Fiske and Subbarow<sup>5</sup> as modified by King<sup>6</sup>. One unit of enzyme activity is defined as  $\mu$  moles substrate hydrolysed in 2.5 ml reaction mixture in 15 min. at  $49^\circ$ . Protein was estimated by the biuret method of Robinson and Hogden<sup>7</sup>.

## Preparation of the Enzyme

5 g of freshly powdered green gram seeds (passing 40 mesh) were extracted in the cold ( $0-5^\circ$ ) with 50 ml of water for six hours with occasional stirring. The extract was centrifuged at 2,500 r.p.m. for 5 min. and the supernatant dialysed against 6 litres of water. It was centrifuged at 3,000 r.p.m. for 20 min. and the supernatant was used as the enzyme preparation.

## Results

**Effect of pH:** The effect of pH on the activity of the crude extract with and without  $Fe^{+++}$  is shown in Fig. 1. In the absence of  $Fe^{+++}$  the enzyme is active only in the acid



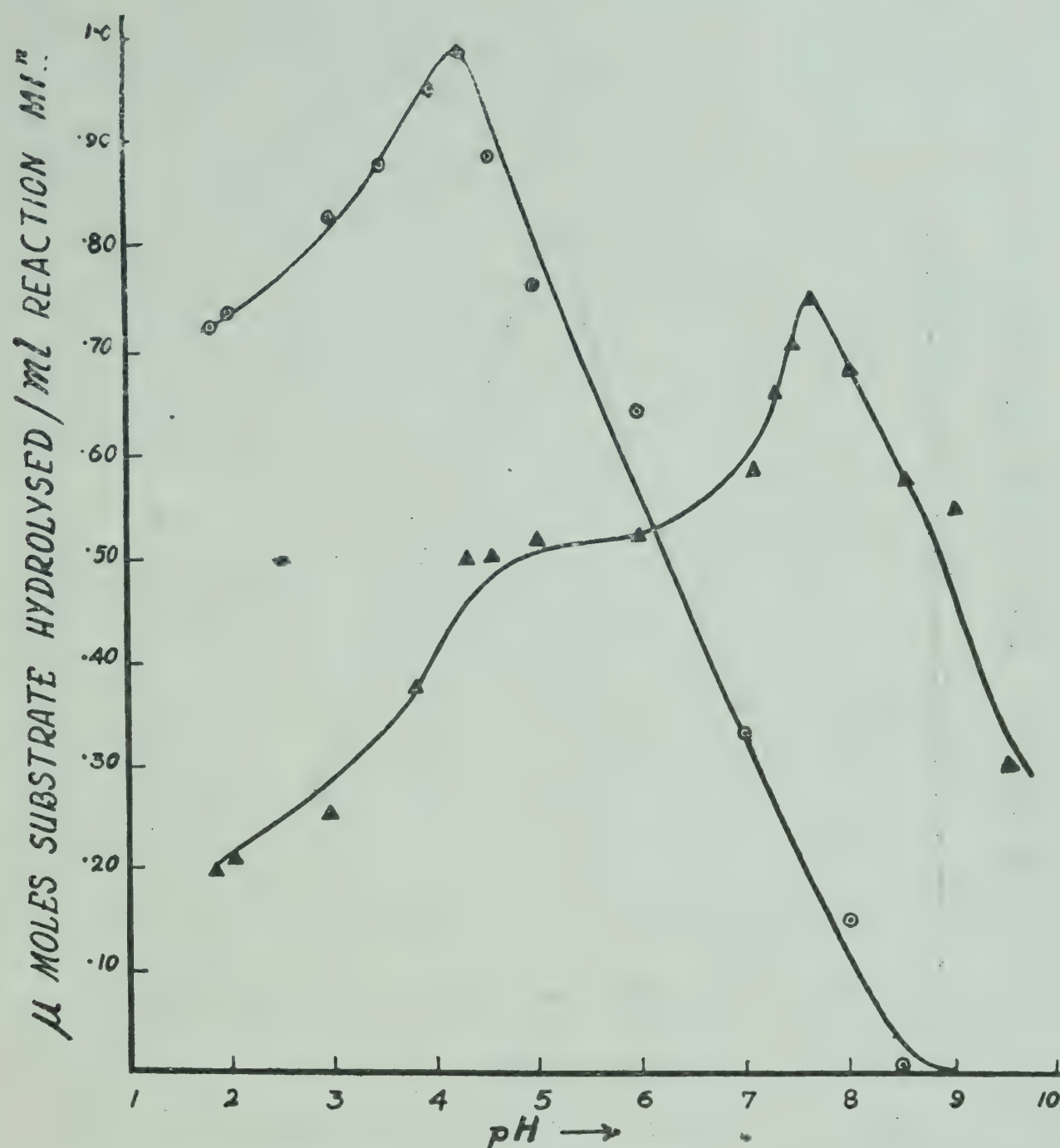


FIG. 1. EFFECT OF pH ON THE CRUDE EXTRACTS OF PHASEOLUS RADIATUS WITH AND WITHOUT  $Fe^{+++}$  ON  $\beta$ -GLYCEROPHOSPHATASE  
 ○-○ without  $Fe^{+++}$  ^ Δ-Δ with  $Fe^{+++}$

range, the maximum activity being at pH 4.3. In the presence of  $Fe^{+++}$ , however, the acid phosphatase activity is inhibited and a new peak at pH 7.7 makes its appearance. Tris, [tris—(hydroxy methyl) aminomethane], glycine-NaOH, boric acid-borax and veronal-acetate buffers were tried at the alkaline pH in the presence of  $Fe^{+++}$  and it was found that Tris and veronal-acetate buffers were the best. Veronal-acetate buffer was used in all the assay mixtures (Fig. 2).

**Effect of temperature:** The rate of hydrolysis was examined over the range 20-100°. The temperature optimum was 49° and rapid inactivation was observed with further increase in temperature.

**Effect of  $Fe^{+++}$  ions:**  $Fe^{+++}$  concentration is a critical factor and the optimum concentration is 2.9  $\mu$  moles/ml, higher concentrations being inhibitory (Table I).

**Effect of metal ions:** In the absence of added ions there was no activity in the alkaline range. In the presence of  $Fe^{+++}$ , heavy metal ions like  $Mn^{++}$ ,  $Cu^{++}$ ,  $Zn^{++}$ ,  $Fe^{++}$  were



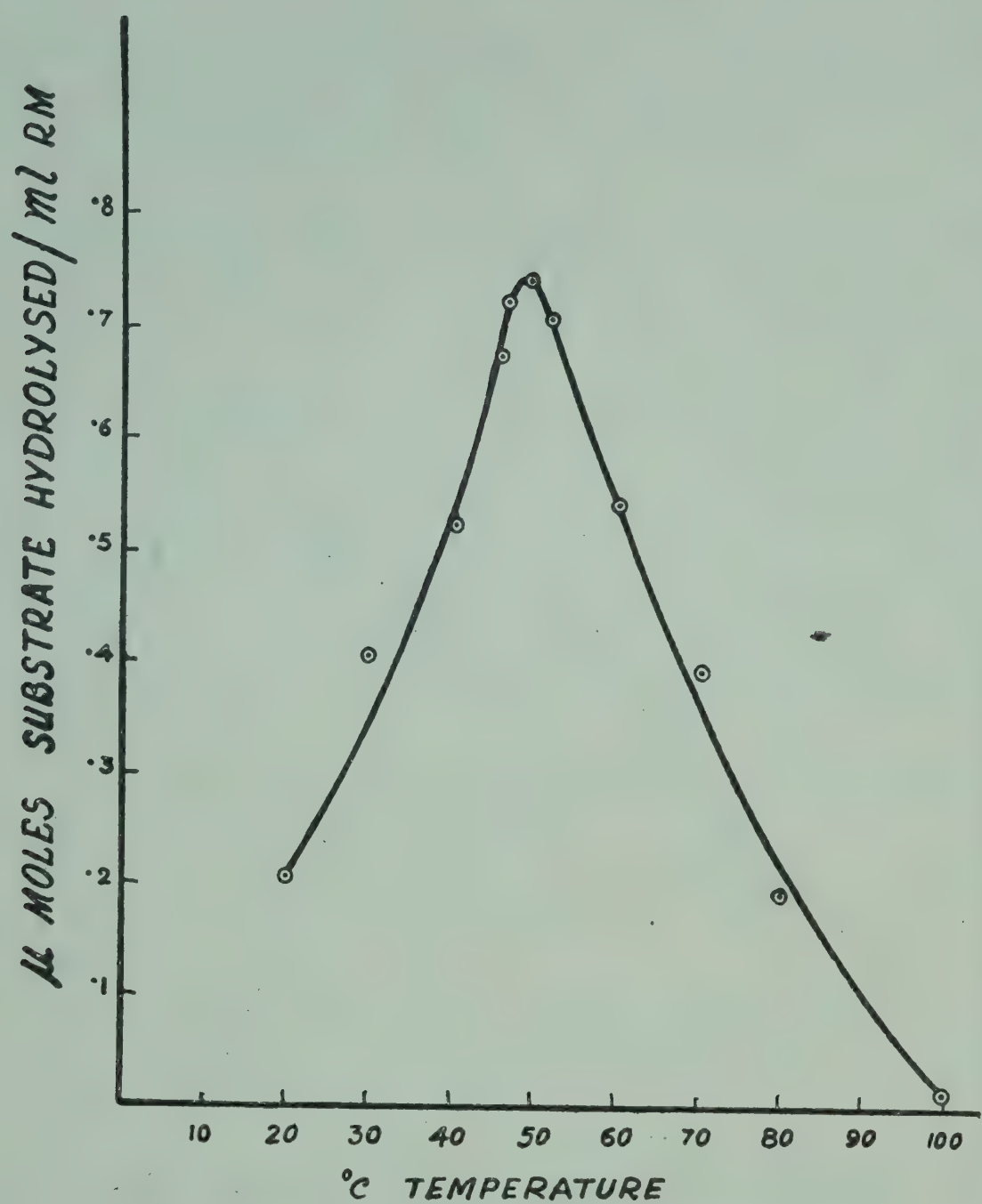


FIG.2. EFFECT OF TEMPERATURE

TABLE I

Effect of Fe+++ concentration on the alkaline β-glycero-phosphatase of green gram

Concentration	Activity*
μ moles/ml.	
0.8	0.13
1.2	0.35
1.6	0.47
2.0	0.60
2.4	0.67
2.9	0.75
3.2	0.63
3.6	0.52
4.0	0.47
4.6	0.38
5.0	0.35
6.0	0.25
7.0	0.15

\* μ moles substrate hydrolysed per ml reaction mixture.



highly inhibitory (Table II).  $Mg^{++}$  which is a well-known activator of alkaline phosphatase of animal tissues depressed the  $Fe^{+++}$  activated phosphatase of green gram.

*Effect of substrate concentration:* The Lineweaver-Burk plot for  $\beta$ -glycerophosphate is given in Fig. 3. The  $K_m$  was calculated to be  $6.25 \mu\text{ moles/ml}$  and the substrate optimum  $17.5 \mu\text{ moles/ml}$ , at a  $Fe^{+++}$  concentration of  $2.9 \mu\text{ moles/ml}$ .

TABLE II  
Effect of various salts on the  $Fe^{+++}$  requiring alkaline  $\beta$ -glycerophosphatase

Substance		Per cent inhibition (–) or activation (+) at concentrations of		
		$10 \mu\text{ moles/ml}$	$1.0 \mu\text{ moles/ml}$	$0.1 \mu\text{ moles/ml}$
Manganese sulphate	...	–36	–22	–18
Copper sulphate	...	–70	–52	–40
Magnesium sulphate	...	–10	+2	0
Cobalt acetate	...	–11	–5	0
Mercuric chloride	...	–64	–48	–35
Zinc sulphate	...	–75	–50	–42
Sodium fluoride	...	–58	–46	–38
Sodium molybdate	...	–90	–85	–80
Nickel chloride	...	–28	–20	–5
Lithium sulphate	...	–15	–12	0

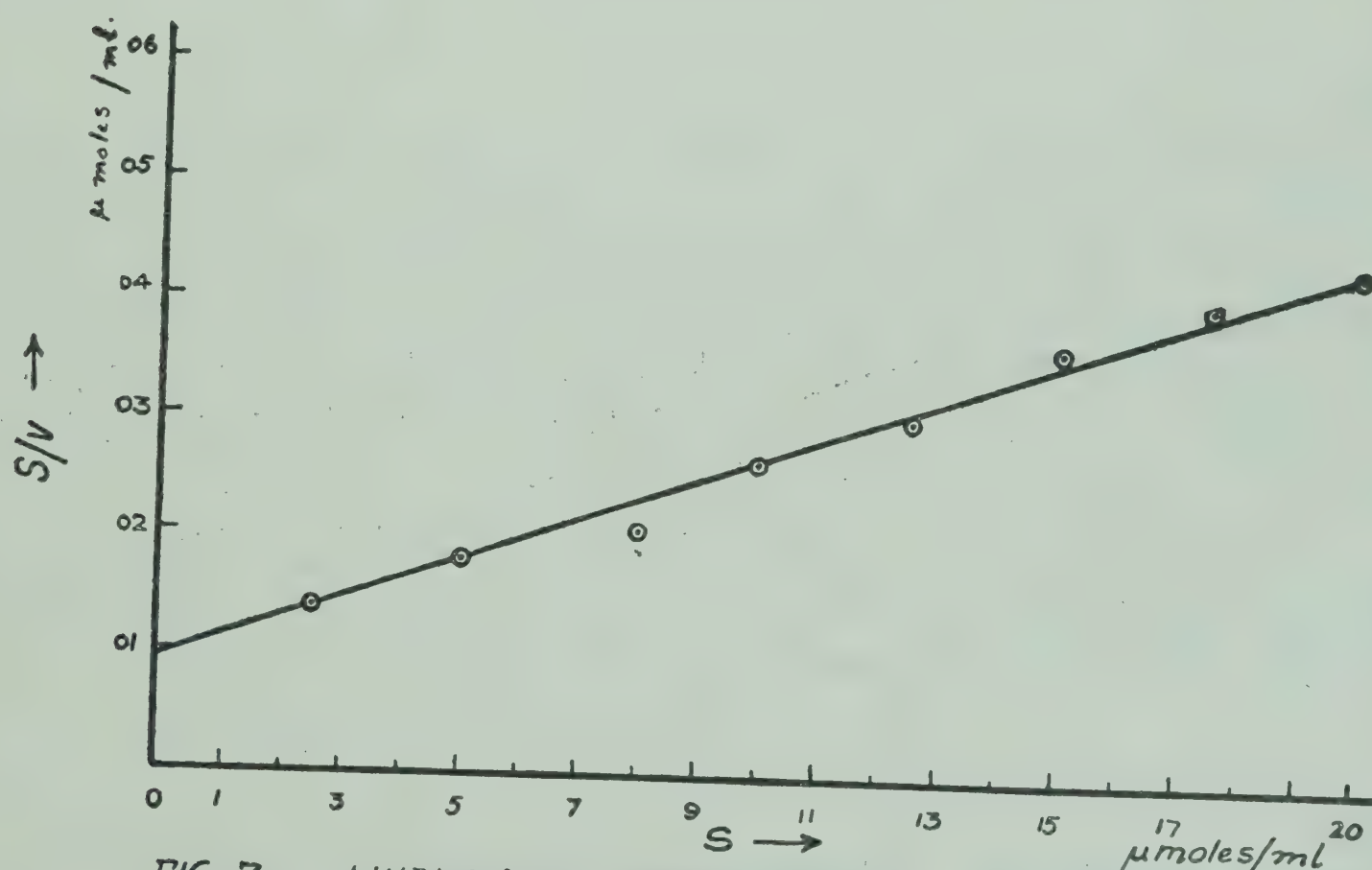


FIG. 3. LINEWEAVER – BURK PLOTS.  $K_m$   $6.25 \mu\text{ moles/ml}$   
SUBSTRATE OPTIMUM  $17.5 \mu\text{ moles/ml}$   
 $S$  = Substrate ;  $V$  = Substrate hydrolysed

*Occurrence of  $Fe^{+++}$ -requiring alkaline glycerophosphatase in plants:* Among the various plant sources tested for enzyme activity (Table III), it was present in almost all of them, the highest activity being in the resting seeds of green gram (*Phaseolus radiatus*) under the conditions specified.



TABLE III  
Occurrence of the  $Fe^{+++}$ -requiring alkaline  $\beta$ -glycerophosphatase  
in plants

Source (Resting seeds)	Activity*
<i>Phaseolus radiatus</i> (Green gram)	0.74
<i>Phaseolus mungo</i> (Black gram)	0.40
<i>Phaseolus vulgaris</i> (French bean)	0.23
<i>Dolichos lablab</i> (Field bean)	0.27
<i>Cicer arietinum</i> (Bengal gram)	0.13
<i>Trigonella sp.</i> (Menthi)	0.20
<i>Vigna catieng</i> (Cow pea)	0.65
<i>Hibiscus esculentis</i> (Ladies finger)	0.25
<i>Brassica campestris</i> (Mustard)	0.44
<i>Coriandrum sativum</i> (Coriander)	0.26

\*  $\mu$  moles substrate hydrolysed/ml reaction mixture at  $49^\circ$ ,  
pH 8.5,  $2.9 \mu$  moles  $Fe^{+++}$ , for 15 min.

**Purification:** An 80-fold purification of the enzyme has been achieved by ammonium sulphate fractionation and isoelectric precipitation.

## Discussion

The enzyme from green gram exhibited maximum activity at a pH of 7.7 in the presence of  $Fe^{+++}$  at a concentration of  $2.9 \mu$  moles/ml. Although the pH optimum is similar to that of the bone alkaline phosphatase and plant alkaline pyrophosphatase, it is not identical with these enzymes, for, it exhibits high specificity with regard to the metal ion requirement. Whereas the alkaline pyrophosphatase of Naganna *et al.*,<sup>3,4</sup> is activated by  $Mg^{++}$ , our preparations are not activated. On the contrary,  $Mg^{++}$  in the presence of  $Fe^{+++}$  inhibits the alkaline phosphatase.

The present findings stress the importance of metal ions like  $Fe^{+++}$  in plant metabolism and also re-emphasise the need for using a variety of experimental conditions while looking for the presence of a particular enzyme in biological systems.

## Summary

An enzyme hydrolysing  $\beta$ -glycerophosphate and requiring  $Fe^{+++}$  ions in the alkaline range has been observed for the first time in plants. The preparation and some of the general properties of the enzyme from green gram (*Phaseolus radiatus*) are described. The enzyme functioned optimally at a pH of 7.7 and at a temperature of  $49^\circ$ . The optimum  $Fe^{+++}$  concentration was  $2.9 \mu$  moles per ml. The  $K_m$  for  $\beta$ -glycerophosphatase was  $6.25 \mu$  moles per ml. The enzyme was found to hydrolyse in the presence of  $Fe^{+++}$ , only  $\beta$ -glycerophosphate and thus differs from bone alkaline phosphatases and alkaline pyrophosphatase of potato. Heavy metal ions were inhibitory.



### Acknowledgment

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# A NATURALLY OCCURRING INHIBITOR OF GLUTAMINE SYNTHESIS PRESENT IN *PONGAMIA* GALLS

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Glutamine and asparagine accumulate in plants either in response to feeding with ammonium salts or during the catabolic reactions which occur in etiolated seedlings or detached leaves<sup>1</sup>. Reversal of catabolism by exposure to light or by exogenous supply of carbohydrates results in utilization of the amide for resynthesis of protein<sup>2,3</sup>. According to current concepts, glutamine and asparagine are considered to be storage reserves of nitrogen, even in systems in which protein breakdown may occur. Recent studies<sup>4</sup> have invested glutamine with a special importance in the nitrogen economy of growing, proliferating, protein-synthesizing cells.

It was, therefore, thought to be of interest to study the mechanism of synthesis of glutamine in the galls present in the leaves of *Pongamia glabra*, as these tissues are characterized by uncontrolled tumorous growth. Oddly enough, preliminary experiments showed not only that the gall extract was devoid of glutamotransferase activity but also that the addition of the gall extract to the standard assay system containing transferase enzyme isolated from dried green peas, effectively interfered with the enzyme reaction. The general properties of this naturally occurring glutamotransferase inhibitor are described in this paper.

## Materials and Methods

*Purification of the transferase: From peas:* Glutamotransferase was purified according to the method of Elliott<sup>5</sup>.

Dry pea meal was extracted with cold 0.1M NaHCO<sub>3</sub> for 30 minutes. 2M MgSO<sub>4</sub> was added with stirring and the precipitate allowed to settle overnight at 0°C. The pH of the supernatant was adjusted to 6.5 and ammonium sulphate (300 g/litre) was added. The precipitate obtained after settling overnight at 0°C was suspended in water and the pH adjusted to 7.2. The suspension was dialysed against cold distilled water for 36 hrs. The enzyme was mixed with phosphate buffer (pH 7.4) and treated with 3/5 its volume of saturated ammonium sulphate solution. After 15 minutes, a further quantity (3/5 its volume) of saturated ammonium sulphate was added to the supernatant. After 20 minutes the residue was separated and redissolved in a small volume of cold water and the pH adjusted to 7.3. The solution was dialysed against frequent changes of cold distilled water. The precipitate formed during dialysis was discarded.

The solution was next treated with 1/10 its volume of 1 per cent potassium nucleate solution and 0.2 M acetic acid. The precipitate was dissolved in 0.01 M phosphate buffer (pH 7.3).

*From sheep brain:* Water extract of the acetone powder of the grey matter of sheep brain was used as the enzyme preparation<sup>6</sup>.

*From Cucurbita pepo:* Seedlings of *C. pepo* (7-9 days) were homogenized with cold acetone. Water extract of the acetone powder was adjusted to pH 5.45 with 2 per cent acetic acid. The supernatant was adjusted to pH 4.8 and the precipitate collected. This was dissolved in 0.05 per cent sodium bicarbonate solution<sup>7</sup>.



*Estimation of glutamotransferase and synthetase activities:* The transferase was assayed in 3.5 ml of the incubation mixture containing 0.1 ml of potassium arsenate (0.1M), 0.1 ml L-glutamine (0.1M), 0.1 ml  $\text{MnSO}_4$  (0.1M), 0.1 ml of 0.002 M. ATP, 0.2 ml  $\text{NH}_2\text{OH}$  (0.1M), 1.4 ml of Tris buffer (pH 7.5), 1.0 ml of the enzyme and 0.5 ml of the inhibitor preparation (or water).

The total volume of the reaction mixture for the assay of the synthetase activity was the same as the transferase but the composition was: 0.7 ml. Tris buffer (pH 7.2), 0.5 ml ATP (0.05M), 0.5 ml glutamic acid (0.5M, pH adjusted to 7.2), 0.1 ml  $\text{MgSO}_4$  (1.0M), 0.1 ml  $\text{NH}_2\text{OH}$  (1.0M, pH adjusted to 7.2), 1.0 ml of enzyme and 0.5 ml of inhibitor or water (cysteine hydrochloride was omitted from the incubation mixture).

Glutamohydroxamic acid formed in both the transferase and synthetase systems was measured colorimetrically by the method of Lipmann and Tuttle<sup>8</sup>.

*Estimation of glutaminase activity:* The reaction mixture for the assay of glutaminase activity contained 0.5 ml Tris buffer (pH 7.5), 0.1 ml  $\text{MgSO}_4$  (0.3M), 0.1 ml, ADP (0.01M), 0.1 ml arsenate (0.25M), 0.2 ml glutamine (0.1M), 1 ml of the enzyme and 0.5 ml of inhibitor preparation or water in a total volume of 2.5 ml<sup>9</sup>.

3 ml acetone was added to deproteinize and the mixture concentrated *in vacuo*. The amounts of glutamine hydrolyzed and glutamic acid formed were estimated by paper chromatography.

*Estimation of ATPase activity:* The ATPase activity of the inhibitor preparation was followed by measuring the amount of inorganic phosphate liberated by the method of Fiske and Subbarow<sup>10</sup>.

Incubation mixture consisted of a total volume of 2.2 ml containing 0.6 ml buffer (Tris pH 7.5), 0.2 ml ATP (1 mg), 0.6 ml enzyme, 0.2 ml inhibitor (or water) and 0.6 ml water.

*Proteolytic activity:* The free amino acid liberated was determined by formol titration<sup>11</sup>.

30 g of casein were triturated with 15 ml of water. 270 ml of water and 16.5 ml of 2 N NaOH were added and the pH was adjusted to 8.1. 80 ml of the casein solution was warmed to 37°C and 5 ml inhibitor preparation added. Amino acid nitrogen was determined by titration against 0.1N NaOH.

## Results

Having ascertained that the aqueous extract of the gall tissue had no effect on the colour reaction of glutamohydroxamic acid with ferric chloride, the question whether the inhibiting factor prevents in some way the formation of glutamohydroxamic acid or destroys the glutamohydroxamic acid as soon as it is formed was next studied. It was found that the addition of gall extract to the standard reaction mixture, containing glutamohydroxamic acid formed by enzyme action, had no effect on the concentration of hydroxamic acid even after prolonged incubation. This suggested that the inhibitor interfered with the formation of glutamohydroxamic acid catalysed by glutamotransferase.

It was observed that the gall residue, after repeated extraction with water still exhibited inhibitor activity. Each of the reaction constituents was tested individually for its capacity to solubilize the inhibitor. Butanol and digitonin treatments were also tried. The results given in Table I show that arsenate, butanol and digitonin treatments effected the solubilization of the inhibitor,



TABLE I  
*Effect of different solubilizing agents*

Treatment		% Inhibition	
		Residue	Supernatant
Digitonin <sup>1</sup>	...	42.66	28.00
Butanol <sup>2</sup>	...	53.12	56.25
Arsenate (0.02M) <sup>3</sup>	...	33.63	36.28
Glutamine (0.02M)	...	32.74	60.18
Manganous sulphate (0.02M)		0	41.60

<sup>1</sup> A suspension of the washed residue in Tris buffer (pH 7.0) was treated with an equal volume of 0.2% digitonin solution (pH 7.0). After 20 min. the residue was separated and the activity of the residue and supernatant determined.

<sup>2</sup> A suspension of the washed residue in 10 ml of water was treated with 5 ml of n-butanol, triturated and centrifuged. Activity of the aqueous layer and that of the residue was determined.

<sup>3</sup> The washed residue was treated separately with arsenate, glutamine,  $\text{MnSO}_4$  centrifuged after 30 min. and the supernatant dialysed before use.

*Properties of the soluble form of the inhibitor:* The inhibitor preparation was found to be thermolabile. The activity decreased gradually up to 60°. Further increase in temperature resulted in marked inactivation. Heating for 15 min. at 100° resulted in almost complete inactivation.

The inhibitor preparation was nondialysable and had little proteolytic activity.

*ATPase activity:* The crude extract of the galls was found to contain ATPase activity. Since ATP is essential for the energy transfer reaction in the system, the inhibition might be expected to result by a destruction of the ATP. Table II shows that the ATPase activity of the preparation was almost completely destroyed by heating at 60° for 120 min. The inhibitor activity was more heat stable.

TABLE II  
*Effect of heat on the ATPase activity of the inhibitor*

Time of heating at 60°		$\mu\text{g}$ phosphorus liberated	% activity
15 min.	...	13	40.65
30 min.	...	12	37.50
45 min.	...	8	25.00
60 min.	...	7	21.56
120 min.	..	3	9.38



The possibility that the inhibitor might be an enzyme which causes the degradation of some of the intermediates in the reaction was excluded by the fact that the inhibitor preparation was stable in 2.5 per cent trichloroacetic acid solution.

TABLE III  
*Stability of the inhibitor in the presence of dilute trichloroacetic acid solution (2.5%)*

System	$\mu\text{g}$ glutamohydroxamic acid formed	% Inhibition
No inhibitor ...	545	
Pea enzyme+inhibitor ...	285	47.71
Pea enzyme+inhibitor (TCA treated) ...	225	58.71

After treating with TCA the inhibitor preparation was dialysed and the pH was adjusted to 7.

The preparation was found to exert the inhibitory activity on the glutamotransferase, synthetase and on the glutaminase activity of the pea enzyme as shown in Tables IV and V.

TABLE IV  
*Action of the inhibitor on transferase and synthetase*

System	$\mu\text{g}$ GHA formed		% Inhibition	
	transferase	synthetase	transferase	synthetase
Pea enzyme ...	600	330		
Pea+gall extract ...	200	155	66.67	53.03
Pea+partially purified inhibitor	110	100	81.67	69.69

TABLE V  
*Action of the inhibitor on the glutaminase*

System	$\mu\text{g}$ glutamic acid formed
Pea enzyme (0 hr) ...	2.2
Pea enzyme ...	3.0
Pea enzyme + inhibitor (0 hr) ...	2.4
Pea enzyme + inhibitor ...	2.8

Table VI shows that the glutamotransferase from different sources, namely, pea, *C. pepo* and sheep brain are inhibited by the inhibitor preparation.

The enzyme was incubated with the inhibitor preparation for different lengths of time. The results given in Table VII show that there is no alteration in inhibitory potency when the enzyme is incubated with the inhibitor.

Table VIII shows the effect of incubation of the inhibitor and the substrate which results in an increase in inhibitory potency.



TABLE VI  
*Comparison of the activity of the inhibitor on transferase from different sources*

Source	$\mu\text{g}$ GHA formed		% Inhibition
	without inhibitor	with inhibitor	
Pea ...	575	250	56.52
<i>Cucurbita pepo</i> ...	430	185	57.00
Sheep brain ...	1850	657	64.49

TABLE VII  
*Effect of incubation of enzyme and inhibitor*

Time of incubation (min.)	% Inhibition
0	51.91
15	51.91
30	54.96
60	54.20
120	54.20

TABLE VIII  
*Effect of incubation of inhibitor and substrate*

Time of incubation (min.)	% Inhibition
0	51.9
15	50.4
30	56.5
60	56.5
120	65.7

Discussion

In this investigation, evidence has been presented for the occurrence of a natural inhibitor of glutamotransferase activity in the galls and normal tissues of *Pongamia glabra*.

The heat lability and non-dialysable nature of the inhibitor suggest that it may be a protein with a high molecular weight.

The lack of correlation between the proteolytic and ATPase activities of the various fractions on the one hand, and the inhibitor activitiy, on the other, clearly rules out the



possibility of experimental artefacts and indicates that it is a true inhibitor of glutamotransferase.

The observation that both the glutamotransferase and synthetase activities are inhibited by the purified preparations of the gall extract supports the view that both these reactions are catalysed by the same protein.

The mode of action of the inhibitor is far from clear. It is possible that the inhibitor, which is most probably a protein, may have multiple catalytic functions and that it has a role in the utilization of glutamine. Another possibility is that the inhibitor might cause the enzymic degradation of some of the intermediates in the reaction. This is, however rendered unlikely by the stability of the inhibitor in presence of dilute trichloroacetic acid. Moreover, no intermediates have been identified in the glutamine synthetase reactions, and quite a number of probable intermediates, like glutamyl phosphate, have been clearly ruled out<sup>12</sup>. Kinetic studies<sup>13</sup> have indicated that any interaction between glutamic acid and ATP prior to the addition of  $\text{NH}_3$  does not involve covalent bond formation.

Because of the rather inchoate state of our knowledge of the mechanism of glutamotransferase action, it would be premature at this stage to speculate on the mode of action of the inhibitor.

The presence of the inhibitor in high concentrations in the gall tissue also presents problems of physiological nature. We have observed that the gall tissue contains appreciable amounts of glutamine and it is difficult to explain how glutamine is synthesized in the presence of the inhibitor. One plausible explanation is that the inhibitor and the enzyme may occur at different intracellular loci. This possibility is now being investigated.

## Summary

The general characteristics of a naturally occurring inhibitor of glutamine synthesis present in *Pongamia* galls have been investigated.

The inhibitor occurs both in 'soluble' and 'insoluble' forms. The 'insoluble' form can be brought into solution by digitonin and butanol treatments and by extraction with a dilute solution of arsenate.

The results indicate that the inhibiting factor is a high molecular weight protein which is stable in presence of 2.5 per cent trichloroacetic acid.

Both the transferase and synthetase activities of glutamotransferase were inhibited by purified preparations of the inhibitor.

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# THE INFLUENCE OF CHELATING AGENTS ON THE TRANSPORT OF COBALT BY BLOOD PROTEINS

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Our previous experiments have shown that the simultaneous administration of chelating agents can profoundly alter the tissue distribution of Cobalt-60 in albino rats. Intravenous injection of tracer Cobalt-60 alone results in an excretion of nearly 80 per cent within 24 hours, mostly through the urine. 1-nitroso, 2-naphthol effectively decreases the excretion to 14 per cent. On the other hand, ethylenediamine tetraacetic acid (EDTA) increases the excretion to nearly 99 per cent, the distribution of the EDTA complex in the various organs being conspicuously low, amounting to less than 1 per cent of the activity injected. Both rubeanic acid and 2:3 dimercapto propanol (BAL) cause a localization of the isotope in the liver (46 per cent and 45 per cent respectively) and sodium diethyl dithiocarbamate in the intestine (40 per cent). Thioglycolic acid produces slightly greater retention in the blood, from 2.5 to 17 per cent. In an attempt to explain these various changes in distribution of cobalt, due to chelating agents we are at present investigating the effects of these chelating agents on the transport of cobalt by serum proteins and preliminary results are reported here.

## Experimental

The experiments involve the injection of radioactive cobalt, alone or with a chelating agent, followed by studying the exact state in which the tracer or the cobalt tracer-chelating agent complex occurs in the blood. For the latter purposes, dialysis of whole blood, trichloroacetic acid (TCA) precipitation of serum, paper electrophoresis of serum, along with radioactivity measurements at each step in the various fractions have been carried out.

In the first series of experiments, Cobalt-58 obtained from the Isotope Division, AEET, Bombay, was used. It was a sterile, carrier-free cobaltous chloride solution containing a little sodium chloride, at neutral pH. When this preparation got exhausted, Cobalt-60, procured from the Radiochemical Centre, England, was used. It was also as cobaltous chloride solution with a high specific activity. 100 microcuries, used for each injection, contained less than 3.3 micrograms of cobalt.

Male albino rats, weighing 250-300 g and divided into groups were used. Each rat in the first group ('the controls') was injected intravenously, through the tail vein under light ether anaesthesia with 0.2 ml of carrier-free Cobalt-58 solution (100 microcuries) plus 0.5 ml of isotonic saline; while the rats in the other groups received the same amount of tracer solution plus 0.5 ml of 5 mM solution of any one of the chelating agents, at or near pH 7.0. A large amount of radioactivity, as high as 100 microcuries per rat, had to be used in order to have enough activity in the 0.01 ml of serum taken later on for electrophoresis. At the end of fifteen minutes after injection, the rats were sacrificed with excess



ether anaesthesia, and 4 ml of blood was removed from the heart. Of this blood, 1 ml was used for dialysis, two 0.4 ml aliquots for radioactivity measurements and the rest for preparing serum. The time of 15 minutes was chosen arbitrarily. It is long enough to allow the injected solution to mix thoroughly with the blood, and yet short enough to prevent most of the radioactivity from leaving the blood compartment.

1 ml of blood in a cellulose bag was dialysed against 40 ml of isotonic saline for 16 hours (overnight) after which time the saline was changed and the dialysis allowed to proceed for 5 more hours. The dialysates were then made up to 100 ml and 5 ml aliquots were used for radioactivity measurements. The cellulose bag containing the dialysed blood was counted separately.

Mixtures of Cobalt-59 and the chelating agents, identical with those used for injection, were separately dialysed against isotonic saline in a similar manner and the activities dialysed out determined. This experiment revealed the behaviour of the cobalt-chelate alone towards dialysis. It was then possible to assess how admixture with blood modified the dialysability.

For measuring the radioactivity of the blood, 0.4 ml of the blood was diluted to 5 ml in a lusteroid tube and then counted in a 'Ekco' Scintillation counter with a 2" well-type NaI-Tl crystal, connected to a 'Ekco' Automatic Scaler. In this set up mainly the gamma or the X-radiation is counted. Suitable corrections were made where the counting rate exceeded 1000 counts per second. The volume of any solution counted was always kept at 5 ml to preserve identical geometry.

From 2.2 ml of blood, serum was prepared by allowing the blood to stand for about half-an-hour and then centrifuging at 1500 r.p.m. for 15 minutes. The sedimented 'cell' fraction was washed twice with isotonic saline and the washings collected separately. Suitable aliquots of the serum, the washings and the cells were counted and the activity in the washings added up to the activity of the serum.

A small aliquot of serum was diluted to 5 ml and 2 ml of 30 per cent TCA added, the precipitate was removed by filtration and washed with 3 per cent TCA. The washings were added to the filtrate and the activities in the precipitate and the filtrate determined.

Paper electrophoresis for the separation of serum proteins was carried out for 16 hours at 250 volts and 7 to 10 milliamps current in a 'Shadon' vertical-type electrophoresis unit in strips of Whatman No. 3 filter paper, 36 cm. long. 10  $\mu$ l of serum was used for spotting and the medium was a 1:1 mixture of veronal and borate buffers, pH 8.6 and ionic strengths 0.083 and 0.1 respectively. At the end of the electrophoresis, the paper strips were dried and then immersed for 5 minutes in 1 per cent bromophenol blue solution in ethyl alcohol saturated with mercuric chloride. The excess of the dye was then washed off, the paper strips again dried, the clear-cut protein bands obtained cut out and counted in the scintillation counter.

## Results and Discussion

The data concerning the dialysis of the various chelates of Cobalt-58 alone, and of blood containing them are presented in Table I. It will be seen that with the exception of 1-nitroso, 2-naphthol and sodium diethyl dithio carbamate complexes, all the others are, for the most part, freely dialysable. While this is quite understandable in the case of 'control', and the soluble EDTA, cysteine and thioglycollic acid chelates, the results



TABLE I  
*Dialysis of Cobalt-58 chelates, alone and in blood*

Chelating agent used		Cobalt-58 plus chelating agent alone		Cobalt-58 chelate in blood	
		Per cent Dialysable	Per cent Non-dialysable	Per cent Dialysable	Per cent Non-dialysable
None (control)	...	86.8	13.2	43.2	56.8
EDTA	...	91.5	8.5	98.3	1.7
1-nitroso, 2-naphthol	...	1.8	98.2	0.8	99.8
Sodium diethyl dithiocarbamate	...	10.2	89.8	25.7	74.3
Oxine	...	76.7	23.3	50.2	49.8
Thioglycollic acid	...	80.4	19.6	22.2	77.8
BAL	...	62.7	37.3	52.9	47.1
Rubeanic acid	...	87.8	12.2	15.5	84.5
Cysteine	...	92.6	7.4	75.3	24.7

with oxine and rubeanic acid, which form thick insoluble precipitates with cobalt at higher concentrations of cobalt, are rather surprising. Even with carrier-free cobalt solutions, as used in these experiments, one could have expected non-penetrating colloidal solutions. Probably, the particle size of the chelate formed is big enough only in the case of 1-nitroso, 2-naphthol and sodium diethyl dithiocarbamate to prevent them from being dialysed out.

The results also reveal that, with the exception of the EDTA and 1-nitroso, 2-naphthol chelates, the dialysability of all the others are considerably modified in blood. The completely dialysable nature of the EDTA chelate strongly suggests that it exists in blood as a free, soluble independent entity unbound to any protein. Probably it does not react with any blood constituent. The considerable reduction in the dialysability of cobaltous chloride (‘the control’), or of most of the chelates, especially those of rubeanic acid and thioglycollic acid, in blood is rather suggestive of appreciable binding of these substances by blood proteins. The question then arises whether the metal chelate is bound as such with the blood protein or whether there is any splitting up of the chelate into the metal and the chelating agent, the metal alone being bound to the protein. The data presented in Table I seem to suggest that, at least in the case of rubeanic acid and thioglycollic acid, the chelate as a whole is bound to the proteins. While the extent of dialysis of cobalt chloride, thioglycollic acid and the rubeanic acid complexes, when alone, are quite comparable (i.e. between 80 and 87 per cent), the extent of dialysis of the two chelates in blood are so low compared to that of cobaltous chloride in blood, that they seem to preclude a splitting up of the chelate in blood. On the other hand, the sodium diethyl dithiocarbamate chelate is probably partially split up when injected into the blood, releasing the metal (Cobalt-58). This would account for the higher percentage of radioactivity dialysed out in blood.



TABLE II

*The relative distribution of radioactivity between cells, serum and serum proteins  
(values expressed as percentages)*

Chelating agent		Blood		Serum	
		Cells	Serum	Serum Proteins (TCA precipitate)	Rest of Serum (TCA filtrate)
None (control)	...	17.4	82.6	74.5	25.5
EDTA	...	29.2	70.8	23.3	76.7
1-nitroso, 2-naphthol	...	54.6	45.4	92.8	7.2
Sodium diethyl dithiocarbamate	...	43.2	56.8	72.5	27.5
Oxine	...	15.6	84.4	67.5	32.5
Thioglycollic acid	...	23.0	77.0	31.8	68.2
BAL	...	23.7	76.3	48.2	51.8
Rubeanic acid	...	36.7	63.3	80.1	19.9
Cysteine	...	22.8	77.2	40	60

In Table II are presented the data concerning the relative distribution of injected radioactivity in blood constituents. It will be observed that in the majority of cases, the greater amount of radioactivity is in the serum. Only in the case of 1-nitroso, 2-naphthol and sodium diethyl dithiocarbamate chelates, the 'cells' fraction has activity nearly equivalent to that of serum. These are also the chelates, whose particle sizes are big enough to give low values for dialysis. The activities in the 'cell' fraction may be due to a small combination of the chelates with the cell proteins, or due to a little serum still adhering to the cells, or most probably due to some of the colloidal chelates sedimenting along with the cells during the centrifugation. The last would seem to be the case with 1-nitroso, 2-naphthol and sodium diethyl dithiocarbamate. The various possibilities have not yet been completely tested out. Similarly, TCA will precipitate down not only the serum proteins, but possibly also some of the chelates which may exist in a colloidal state in the serum, though not protein-bound. The values given under 'TCA—filtrate' may be more unequivocal for the free chelate existing in true solution. It is interesting to note that the 1-nitroso, 2-naphthol complex is almost completely precipitated down by TCA. The data also reveal that a large percentage of the cysteine, thioglycollic acid and the EDTA-complexes exists in the free state.

The results obtained by electrophoresis of serum are presented in Table III. It will be observed that in no case the total activity bound by the serum proteins exceeds 60 per cent, indicating thereby that at least 40 per cent of the chelates exists in the free state in the serum. Most of the binding is done by the albumin and the  $\alpha$ -globulin. Cobalt-58, injected in the ionic form, is protein-bound to about 60 per cent, mostly with the albumin. This is in conformity with the dialysis and the TCA-precipitation data, and also with the earlier findings of Horst<sup>2</sup> in humans and mice. A negligibly low value (5 per cent) is obtained for the protein-binding of the EDTA-complex. This again confirms that this complex almost completely exists as such. Presumably, it is directly excreted from the blood stream without reacting with any of the body constituents. This will account for the 95-99 per cent excretion observed. It is very interesting to note the preferential binding of the thioglycollic acid complex to the  $\alpha$ -globulin, which is in



TABLE III

*Distribution of radioactivity among serum proteins. Values expressed as percentage of the activity (of serum) spotted*

Chelating agent		Albumin	Globulins			Total amount bound to serum proteins
			Alpha	Beta	Gamma	
None (control)	...	26.8	19.9	9.9	1.8	58.4
EDTA	...	2.4	2.6	0	0	5.0
1-nitroso, 2-naphthol	...	7.1	8.8	14.7	0	30.6
Sodium diethyl dithiocarbamate	...	14.9	0	0	7.5	22.4
Oxine	...	21.4	8.2	3.0	2.3	34.9
Thioglycollic acid	...	10.4	34.0	6.5	2.4	53.3
BAL	...	12.2	3.0	1.8	3.3	20.3
Rubeanic acid	...	7.8	13.0	12.8	3.1	36.7
Cysteine	...	12.0	10.5	6.0	0	28.5

sharp contrast to the 'control' (the ionic form), where the most preferred protein is the albumin. This difference in the pattern of distribution again suggests that the chelate as a whole is bound to the protein and that a splitting of the chelate is not a necessary criterion for protein-binding. All the other chelates seem to exist partly free and partly protein-bound.

We have observed that the dialysis of blood containing the thioglycollic acid complex is not complete even after 96 hours. Appreciable amounts of radioactivity are removed indefinitely, though slowly and in small quantities, on prolonged dialysis. This suggests a reversible equilibrium between the free and the protein-bound states and that the binding is labile. It is quite conceivable that similar equilibria exist in the case of the other chelates also.

### Acknowledgment

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# PROTEIN METABOLISM IN UREMIA

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During uremia there is an unphysiological rise in the levels of a number of organic and inorganic substances in the body fluids. Every such substance has been suspected to be responsible for the toxicity of uremia. However, several authors<sup>1,2,3,4,8</sup> have stressed the significance of the non-protein nitrogen elevation in the blood during uremia. Urea is the most abundant component of the non-protein nitrogen (NPN) fraction. It has been shown by Strauss and Raisz<sup>1</sup> and Marshall<sup>2</sup> that urea is relatively innocuous to man. The administration of urea to a healthy person may raise the blood level, but the syndrome of uremia does not appear. Moreover, the other nitrogenous products of protein degradation, viz., guanidine, phenolic compounds and conjugated hydroxy acids were found to be non-toxic at their uremic concentrations by Schmidt *et al*<sup>5</sup>. There is very little information in the literature regarding the increase of free amino acids of the NPN fraction, and the possible toxic effect of the elevated levels of such amino acids during uremia.

It was, therefore, considered that a study of the free amino acids in the plasma of uremic patients might prove useful in elucidating the chemical nature of uremia. Four amino acids, viz., glycine, proline, methionine and arginine, were investigated. Glycine is a toxic amino acid at elevated levels as mentioned by Doolan *et al*<sup>6</sup>. They studied the renal tubular response to amino acid loading and showed that the intravenous administration of glycine caused severe toxic effects in human beings. Arginine is a member of the ornithine cycle, and as such, its fluctuation may occur during uremia. The level of methionine was also suspected to be altered as it occupies a key position in transmethylation processes. Proline was selected for study because proline oxidase is found only in the kidneys, which get damaged in uremia, and consequently the proline level might also change.

## Experimental Materials and Methods

Quantitative paper chromatography was employed for the determination of the free amino acids in the plasma. Blood was collected from five uremic patients who were hospitalized for the treatment of uremia.

**Deproteinization:** Plasma was separated from these samples and was stored at 0°C. These samples had to be liquified by bringing to room temperature before processing. The proteins were precipitated from the plasma by adding absolute alcohol according to the method described by Block<sup>7</sup>. Absolute alcohol was added in such proportion that the final alcohol concentration after mixing was 80 per cent by volume. The bulky white precipitates of proteins were centrifuged and separated. The precipitates were washed twice with 80 per cent ethyl alcohol (v/v) and the precipitates were rejected.

**Concentration:** The alcoholic solution of the free amino acids, as obtained above, was evaporated under reduced pressure at 40° to 45°C. The residue was washed with



five ml of chloroform to remove fatty impurities. The final volume of this concentrated solution was so adjusted with water that its ratio to the original plasma was exactly 1:10. A few drops of 6 N hydrochloric acid were added to it to increase the solubility of the free amino acids.

*Qualitative paper chromatography:* An insulated 'Chromatocab' was used for developing the paper chromatograms. Ten large sheets (56×45 cm) of Whatman No. 1 filter paper could be placed at a time in the chromatocab. The papers were placed for equilibration overnight.

*Densitometry and planimetry:* The colour of the spots was developed with 0.2 per cent ninhydrin solution in absolute alcohol. The proline colour was developed by using 0.3 per cent isatin instead of one per cent isatin solution recommended by Hackman and Lazarus<sup>9</sup>. The density of colour on the spots was read on the Welch Densicron-Densitometer. Three methods of quantitative estimation, namely, (i) the density method, (ii) the area method and (iii) the product of area and density method, were tried for comparison of accuracy and the last one was finally chosen. The area of the spot was marked by pencil with transmitted light. The transmitted light revealed the boundary of the spot more distinctly than the reflected light. The area was measured by a planimeter.

*Standard curves:* Standard curves of individual amino acids were prepared by using five microliter quantities of the standard amino acid solutions ranging from 0.002 M to 0.01 M. Suitable volumes of plasma filtrates to provide concentrations of amino acids in this range were applied on the paper, keeping the area of application as small as possible.

## Results

The results of the amino acid determination in normal and uremic plasma are given in Table I. The normal plasma was pooled from five normal persons. The relative differences between the average of normal and uremic plasma values are diagrammatically represented in Fig. 1. The concentration of free glycine remained at very high levels in the uremic plasma. The levels of arginine and proline were also higher than the normal. The methionine concentration in the uremic plasma was lower than that in the normal.

TABLE I

*Concentration of free amino acids in uremic and normal plasma (microgram per ml)*

Amino acid		Normal plasma (average)	Uremic plasma of patients					Average of five uremic plasma
			1	2	3	4	5	
Glycine	...	12.5	24	59	67.5	47.0	15.0	40.6
Methionine	...	20.0	7.1	10.7	8.0	6.1	8.7	8.1
Proline	...	14.2	18.8	25.2	41.0	12.6	39.0	27.3
Arginine	...	15.2	22.5	18.0	17.9	9.0	19.0	17.3



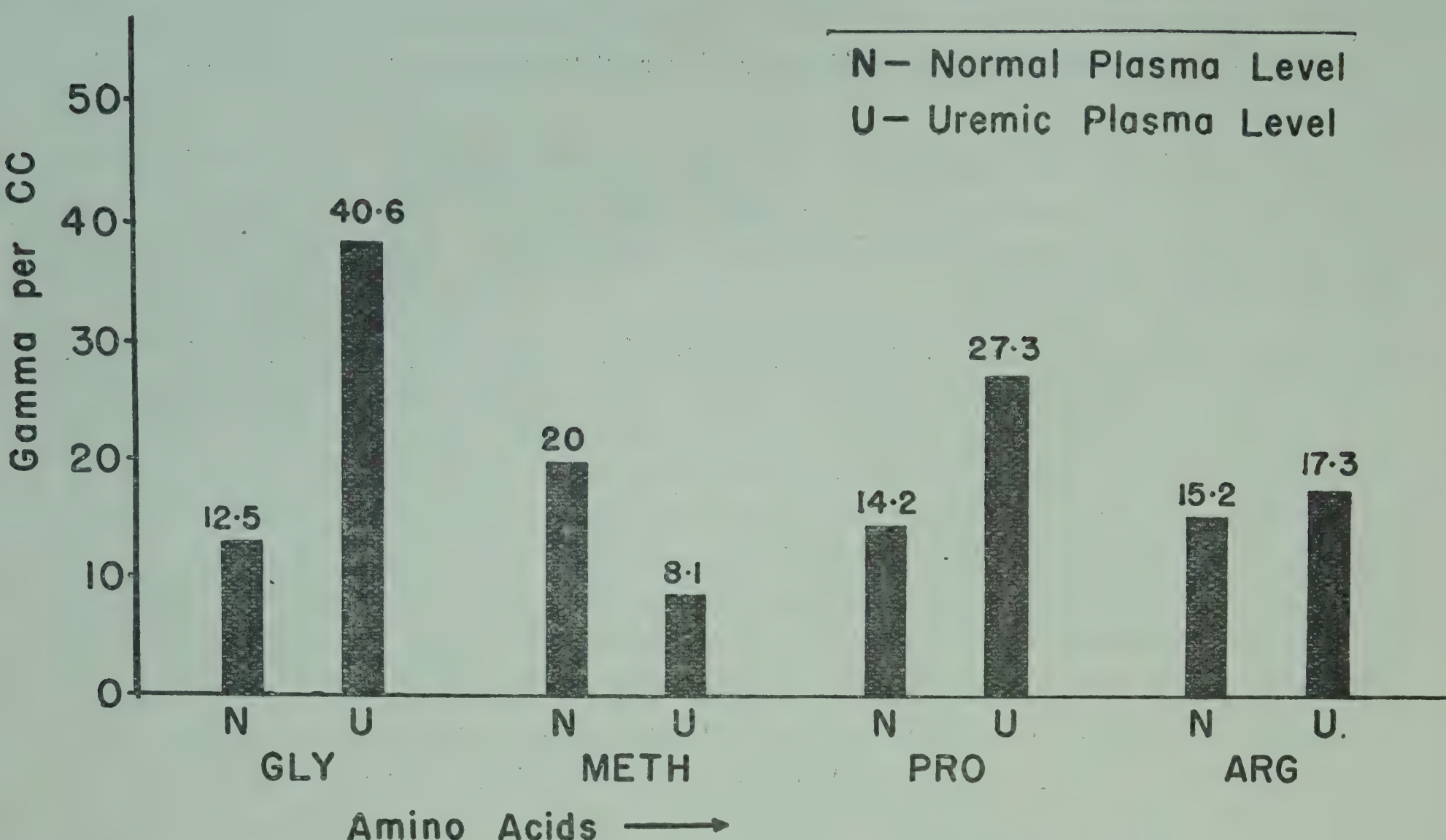


FIG. 1

## Discussion

Doolan *et al.*<sup>6</sup> reported toxic effects after injection of glycine to human beings. They also pointed out that the increase in the levels of one amino acid in the blood affected the levels of the other amino acids. In this study, the glycine values in the four uremic patients are two to five times the normal. A continued higher blood level of glycine for a long period might result in the manifestation of toxicity. The glycine level of the fifth patient was not very high. She was pregnant. The glycine level might not have gone very high due to the metabolic changes in pregnancy. The raised glycine level in uremia might be due to its non-utilization. Glycine is essential for the synthesis of haemoglobin, and anaemia is common during uremia. It is, therefore, probable that the non-utilization of glycine resulted in lower synthesis of haemoglobin.

The higher levels of arginine may be due to: (i) the accumulation of urea and (ii) the dysfunction of the ornithine cycle in the uremic condition. The inhibition of arginase due to accumulation of salts etc., might also build up the arginine level. The increase in proline levels during uremia could be the result of the impairment of the Krebs cycle. Hamburger,<sup>10</sup> reported the dysfunction of the Krebs cycle in the renal failure of uremia. In such cases, if alpha-keto glutaric acid accumulates, its transaminated product, glutamic acid, might also increase. As the synthesis of proline is closely linked up with these two acids, proline might also increase. Moreover, proline oxidase is so far found in the kidney only and the damage to the kidneys in uremia might result in a deficiency of proline oxidase. This might explain the build-up of proline in uremia.

The decrease in free methionine in uremic plasma was considerable. As mentioned above, alteration in the level of one amino acid affects the levels of other amino acids also.



Methionine level might have decreased because of the increase in certain other acids. It should be noted that these uremic patients were on low protein diets. If they were on a normal diet, their blood levels of glycine, proline and arginine might have been still higher. If the comparisons had been made with plasma drawn from normal persons on low protein diet, differences in amino acid values would have been more significant.

### Summary

1. Glycine, arginine, proline and methionine contents of deproteinized normal and uremic plasma were estimated by paper chromatography.
2. Glycine, arginine and proline contents in uremic samples were higher than those in normal blood. Free methionine in uremic plasma was lower than the normal one.

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# EFFECT OF IRON CHLOROSIS ON PROTEIN FRACTIONS OF CORN (*ZEA MAYS*) LEAF TISSUE\*

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Leaf is made up of heterogeneous substances. The separation of a single component in pure form, therefore, involves many problems. In all plant tissues the location of protein relative to the architecture of the cell presents extraction problems. Apart from the interest in the physical, chemical and enzymic properties of cytoplasmic proteins, leaf proteins have been studied in relation to the localization of enzymes and metals in different types of particulate matter inside the cell.

Numerous studies of serum protein patterns have been made in medical science to characterize various pathological conditions. It was therefore thought that similar studies of diseased leaves might yield fruitful information. The present investigation is an attempt in this direction. The effect of iron chlorosis on the pattern of protein distribution on corn leaf tissue is considered here.

## Materials and Methods

Corn plants (*Zea mays*) were raised in solution culture (Hogland's No. 2 formula) under controlled conditions of nutrient levels, reaction of the growth medium, light intensity, humidity and temperature.

There were two treatments in the experiment, one with iron and the other without iron. Five jars with two plants in each were used for the two treatments. In all, six such replications were run. The total number of plants for sampling leaves in each treatment per replication was thus  $5 \times 2 = 10$  plants.

Three weeks after transplanting, leaf samples were collected. Before sampling, plants were removed to a cold room and three leaves, the second, third and fourth from the top of each of the ten plants were harvested and pooled. The surface dust if any was wiped off by means of a clean sponge.

Careful consideration was given for proper sampling of leaf tissue. Four inch portions from the tip and base of the leaves were removed along with the midrib and the remaining part was made into small segments approximately one-half inch in length. Out of the composite sample, 25 g was weighed on a torsion balance and preserved in deep-freeze.

The leaf sample was then divided into half by weighing. The leaf tissue was allowed to thaw at room temperature for 15 minutes and then taken to the cold room. The sample was transferred to a chilled stainless steel jar (200 ml) lined with Teflon. The leaf tissue was allowed to soak in 50 ml water (1:4) for five minutes. It was then homogenised for  $1\frac{1}{2}$  minutes in an 'omnimixer' (Servall model OM) operated at full speed. The jar was kept in crushed-ice bath to prevent rise in temperature during homogenisation. The slurry was filtered through four folds of cheese cloth.

\* The work reported here was carried out at the Utah State University, Logan, U.S.A.



The other half of the leaf tissue was also treated likewise. The homogenates from the two were mixed and subjected to fractionation by differential centrifugation. Relative centrifugal forces upto 17000 xg were obtained using Servall type SS-3 super-speed centrifuge with remote speed control. The centrifugation was done in the cold room. 50 ml Nylon tubes were used in the rotor head. For higher centrifugal forces, the refrigerated Spinco model L ultracentrifuge with rotor head No. 40 was used. In this case 10 ml celluloid tubes were employed. The fractionation procedure employed is summarised schematically in Fig. 1.

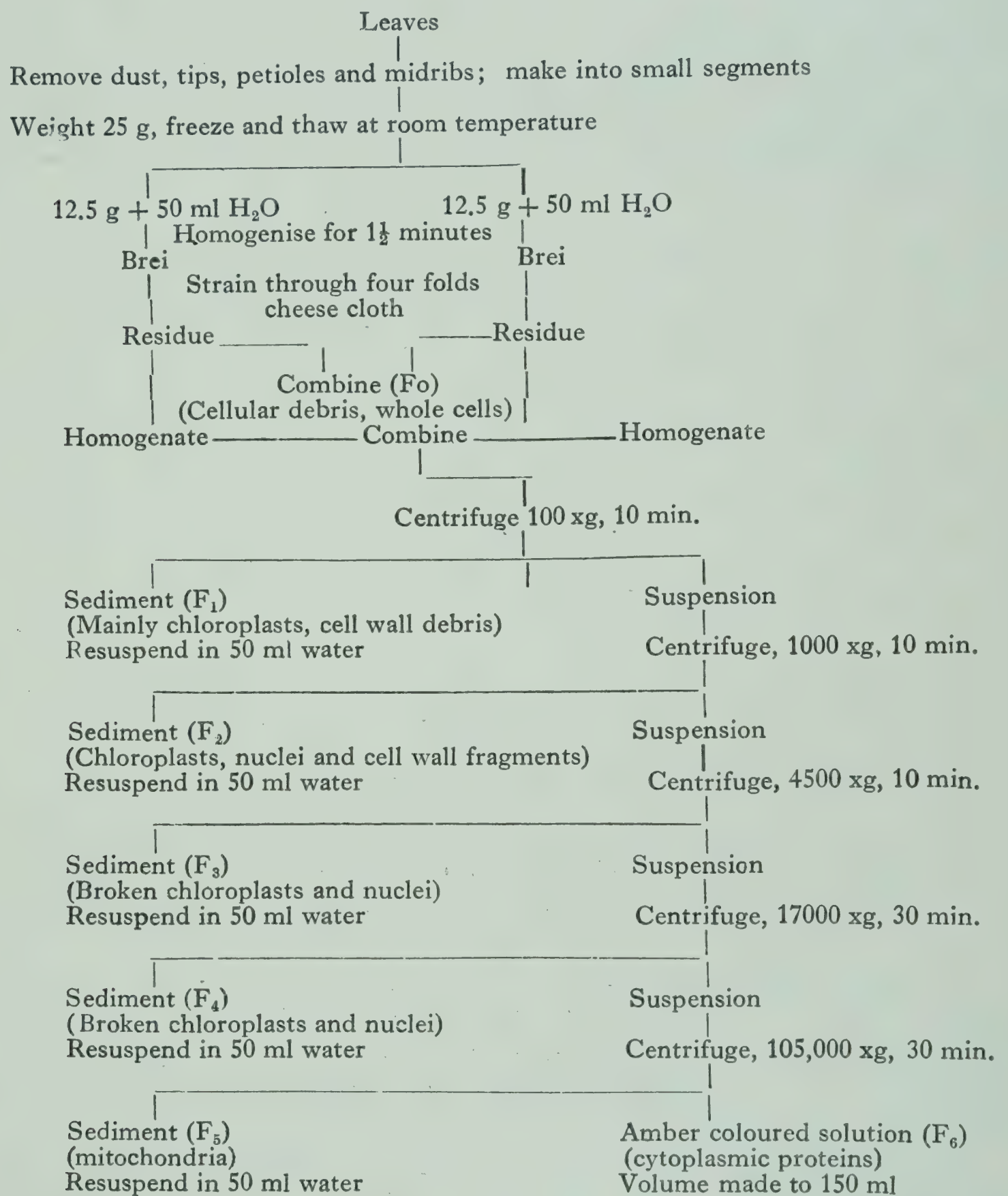


Fig. 1. Flow sheet for fractionation of proteins from corn leaf tissue

After spinning the homogenate at the appropriate speed, the supernatant was transferred to another tube by means of a mechanical pipette for successive centrifugation. Each sediment was washed once with water and recentrifuged, transferring the wash to the



succeeding fraction. The pellets obtained in different fractions were resuspended in water by homogenising in a Ten Broeck homogeniser, making up the final volume to 50 ml. The clear supernatant obtained in the last fraction was, however, made to 150 ml.

Measurement of protein in different fractions was made adopting the Folinpheno reagent method<sup>7</sup>. In all cases the protein fractions were diluted such that one ml of the sample was used for colour development with the reagents, keeping the final volume to 6.5 ml and optical density not higher than 0.5 when read at 650 m $\mu$  on Beckman model B spectrophotometer.

## Results and Discussion

In addition to the protein analysis, total nitrogen in the leaves was also determined by the Kjeldahl method. In Table I, are given the nitrogen and protein contents of the two sets of leaf samples.

TABLE I  
*Nitrogen and protein in normal and chlorotic leaves*

Rep. No.	Normal leaves			Rep. No.	Chlorotic leaves		
	Protein	N	Prot./N		Protein	N	Prot./N
	per cent on dry wt				per cent on dry wt		
1	29.46	4.26	6.91	1	21.10	3.94	5.35
2	34.15	4.49	7.61	2	22.78	4.10	5.55
3	28.96	4.42	6.55	3	25.17	4.20	5.99
4	32.45	4.24	7.65	4	23.67	4.08	5.80
5	32.40	4.29	7.56	5	25.14	4.07	6.18
6	...	...	...	6	...	...	...
Mean	31.48	4.34	7.26	Mean	23.57	4.08	5.77

No appreciable differences was noticed in the nitrogen content of normal and chlorotic leaves. The Kjeldahl method includes both protein and non-protein nitrogen. The slightly lower nitrogen content observed in the case of chlorotic leaves may be explained as due to the reduction in the synthesis of chloroplastic proteins in the absence of iron supply.

In contrast with nitrogen, protein levels for chlorotic leaves are far below those of normal leaves. The reduction is almost 25 per cent. In spinach leaves, nitrogen in the form of chloroplastic proteins makes up roughly 40 per cent of the total nitrogen<sup>3</sup>. Since the chlorotic leaves were deficient in chloroplasts, the protein level became low. It appears from the work of Bennett<sup>2</sup> that iron which is active in chlorophyll formation is that bound in organic combination actually in the chloroplasts and is essential for initiating synthesis of chloroplastic proteins in leaves.



Iron deficiency has, therefore, suppressed the protein synthesis in chlorotic leaves. However, due to the sufficient supply of nitrogen in the culture solution, its absorption continued in spite of the low demand in leaves for protein synthesis resulting in the accumulation of non-protein nitrogen in chlorotic leaves. This is indicated by a low protein to nitrogen ratio in the case of chlorotic leaves when compared with the normal ones. Similar observations have also been made by several workers including Bennett<sup>2</sup> Iljin<sup>6</sup> DeKock and Morrison<sup>4</sup> and Bennett<sup>2</sup> reports that iron deficiency leads to an increase in the amino acid nitrogen and a decrease in protein nitrogen. DeKock and Morrison<sup>4</sup> correlated free amino acids with P/Fe ratio in leaves, the former being high when the ratio is high and declined as the ratio declined. This, they say, is true irrespective of the cause of chlorosis. In the present experiment also, the P/Fe ratio is higher (not reported here) in chlorotic leaves than in the normal ones. This might have contributed to the presence of non-protein nitrogen in higher concentration in chlorotic leaves.

Iljin<sup>6</sup> has reported accumulation of free amino acids in chlorotic leaves. Rhodes *et al.* (1959) have noticed increased aspartic and glutamic acids in bean leaves suffering from chlorosis.

In order to identify the fractions, the preparations were examined under a phase microscope. For this purpose the sediments in different fractions were suspended in water by gentle shaking. Using oil-immersion lens the particles in the prepared suspensions were examined, the description of which is given below (for the description of the fractions  $F_0$ - $F_6$ , refer to the flow sheet for fractionation of proteins from leaf tissue):

- $F_0$ : Mostly cell wall fragments, but few broken cells as well as whole cells were also present.
- $F_1$ : Plenty of chloroplasts could be identified. Most of them were found intact. Some of the broken chloroplasts were also quite large in number. However, these were found slightly damaged at the edges but not completely torn.
- $F_2$ : Few whole chloroplasts and many broken chloroplasts and also nuclei could be observed. Under phase, nuclei were identified as bright spots while chloroplast mass was dark.
- $F_3$ : Few broken chloroplasts, nuclei and other particles suspected to be starch grains.
- $F_4$ : Small particles mostly nuclei like but exhibited vigorous Brownian movement.
- $F_5$ : Rod shaped and sphere like bodies, probably mitochondria.
- $F_6$ : No definite particles could be seen. This fraction when centrifuged was clear amber colour solution. But at the bottom of the centrifuge tube there was a loose viscous mass which could not be separated from the clear solution by a mechanical pipet, because the boundary was not sharp. Even after recentrifuging, this mass did not settle in the form of pellet. When examined under phase microscope, it appeared like a mass of undifferentiated material. There were no individual particles.



TABLE II  
*Protein in corn leaf fractions*

Replication		Normal						Av.	Chlorotic						Av.
		1	2	3	4	5	6		1	2	3	4	5	6	
Fractions		per cent on dry weight													
F <sub>1</sub>	...	2.54	2.92	2.97	2.86	2.75	2.86	2.82	0.57	0.54	0.49	0.37	0.49	0.54	0.50
F <sub>2</sub>	...	0.86	0.92	0.74	0.69	0.51	0.77	0.50	0.63	0.74	0.54	0.48	0.57	0.54	0.56
F <sub>3</sub>	...	0.83	0.86	0.87	0.51	0.34	0.62	0.62	0.51	0.51	0.57	0.66	0.54	0.49	0.55
F <sub>4</sub>	...	0.82	0.86	0.37	0.67	0.63	0.69	0.66	0.57	0.54	0.31	0.37	0.43	0.48	0.45
F <sub>5</sub>	...	0.38	0.37	0.17	0.15	0.11	0.30	0.25	0.09	0.23	0.20	0.23	0.23	0.23	0.20
F <sub>6</sub>	...	3.95	3.55	3.46	3.77	3.03	3.46	3.37	3.40	3.43	2.95	3.11	3.20	3.29	3.23
Total	...	9.38	9.48	8.28	7.74	7.37	8.70	8.47	5.77	5.82	5.06	4.90	5.46	5.57	5.58

Coming to the results of protein analysis of various fractions (Table II), there is a gradual decrease in protein from F<sub>1</sub> to F<sub>5</sub>. However, fraction six (F<sub>6</sub>) contained the maximum amount. The pattern of protein distribution is not the same in both the types of leaves. It is interesting to note that while there is an appreciable difference in protein content of the corresponding fractions of the two kinds of leaves from F<sub>1</sub> to F<sub>5</sub>, very little difference is noticed in the last fraction, F<sub>6</sub>. Compared to 82 per cent reduction in F<sub>1</sub> fraction of chlorotic leaves, the F<sub>6</sub> fraction has suffered a change of only 4.2 per cent on the basis of the corresponding values of the two fractions in normal leaves.

While the rest of the fractions from F<sub>1</sub> to F<sub>5</sub> were the sediments of solid particles, F<sub>6</sub> fraction represented the soluble proteins of the leaf. This many investigators consider as the cytoplasmic proteins. In Table I it was noticed that Protein/N ratio in chlorotic leaves was lower than in normal leaves. This was attributed to the proportionately large amounts of soluble nitrogenous compounds not utilized in the synthesis of conjugated proteins.

Gradual decrease in protein content in other fractions of normal leaves is mainly due to the decrease in the bulk of the sediment obtained at different centrifugal speeds. It is not possible to infer from this data the actual protein content of particles representing these fractions. The figures in Table II show the relative distribution of these fractions in the whole leaf.

The first fraction in the healthy (normal) leaves mainly consisted of chloroplasts as evidenced by the fact that F<sub>1</sub> of chlorotic leaves (naturally deficient in chloroplasts) has only 0.5 per cent protein, while the F<sub>1</sub> of normal leaves contains 2.82 per cent, which is almost 82 per cent more.

Another interesting feature is that there is a sudden fall in protein content from F<sub>1</sub> to F<sub>2</sub> of normal leaves; on the contrary, in the case of chlorotic leaves there is a significant increase, no matter how small, in protein of F<sub>2</sub> and F<sub>3</sub> compared with F<sub>1</sub> fraction. This is



again another evidence for the fact that most of the chloroplasts were sedimented in the first fraction of healthy leaves; chlorotic leaves being deficient in chloroplasts their corresponding  $F_1$  fraction shows lower protein content than  $F_2$  and  $F_3$  fractions.

The trend of results in these experiments is more or less the same as in the corresponding fractions in McClendon's work<sup>8</sup>. Although in this investigation exactly the same centrifugal forces as those used by Gordon<sup>5</sup> were employed, the two results do not agree. An agreement is noticed only with  $F_6$  fractions, but the rest of the fractions are not comparable. This may be attributed to the differences in density of the medium used. Gordon used 0.2M phosphate buffer in 0.3M sucrose solution.

No reference seems to be available in literature on the effect of iron chlorosis on the pattern of protein fractions in leaf tissue. It is, therefore, not possible to compare the results reported here.

In leaf fractions the cytoplasmic proteins did not suffer much quantitatively. Since no appreciable change in cytoplasmic proteins is noticed as a result of chlorosis or iron deficiency, it is suggested that further fractional studies by electrophoresis may show up possible differences in the make up of these proteins.

## Summary

An experiment was conducted to study the effect of iron chlorosis on the pattern of protein distribution in corn leaf tissue. Corn plants were raised under controlled conditions in solution culture and the leaf samples collected from normal and chlorotic plants were analysed for protein and nitrogen contents. Leaf homogenate prepared from the samples were subjected to fractionation by centrifugation at varying speeds. The fractions separated thus were analysed for their protein content.

From the results of the analyses it was observed that there was no appreciable difference in nitrogen content of the normal and chlorotic leaves. However, a reduction to the extent of 25 per cent was noticed in the protein content of chlorotic leaves. Protein-nitrogen ratio in chlorotic leaves was found to be lower than in healthy ones.

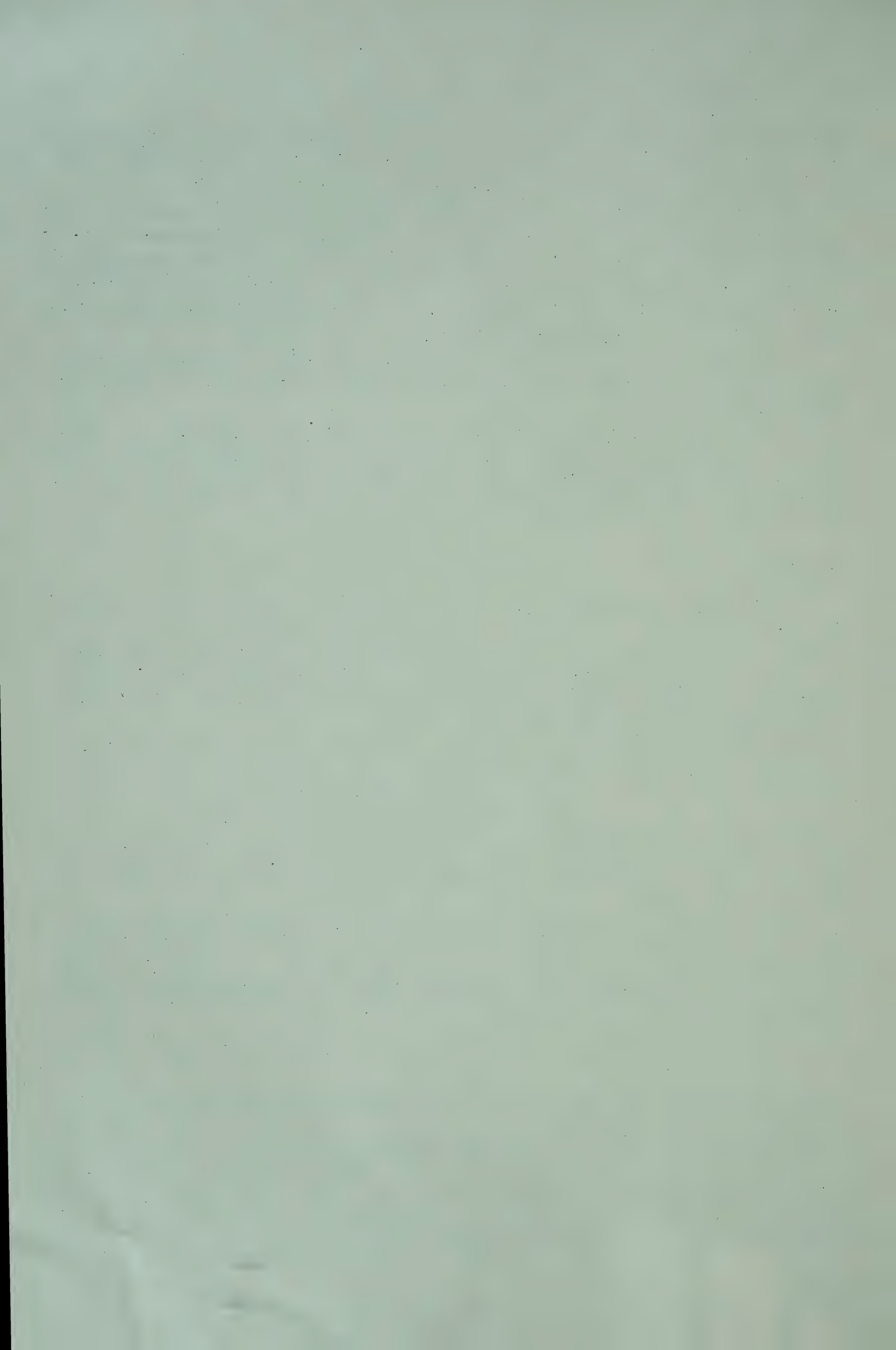
The fractions were examined under a phase-contrast microscope. Chloroplasts were found mainly in  $F_5$  fraction and nuclei in  $F_1$  and  $F_2$  fractions while mitochondria were identified in  $F_3$  fraction.

Protein distribution in the various fractions was not the same in the two kinds of leaves. While there was an appreciable difference in protein content of the corresponding fractions from  $F_1$  to  $F_5$ , in the two kinds of leaves, very little difference was noticed in the last fraction— $F_6$  (cytoplasmic proteins). In chlorotic leaves, the first fraction (chloroplasts) suffered the most.

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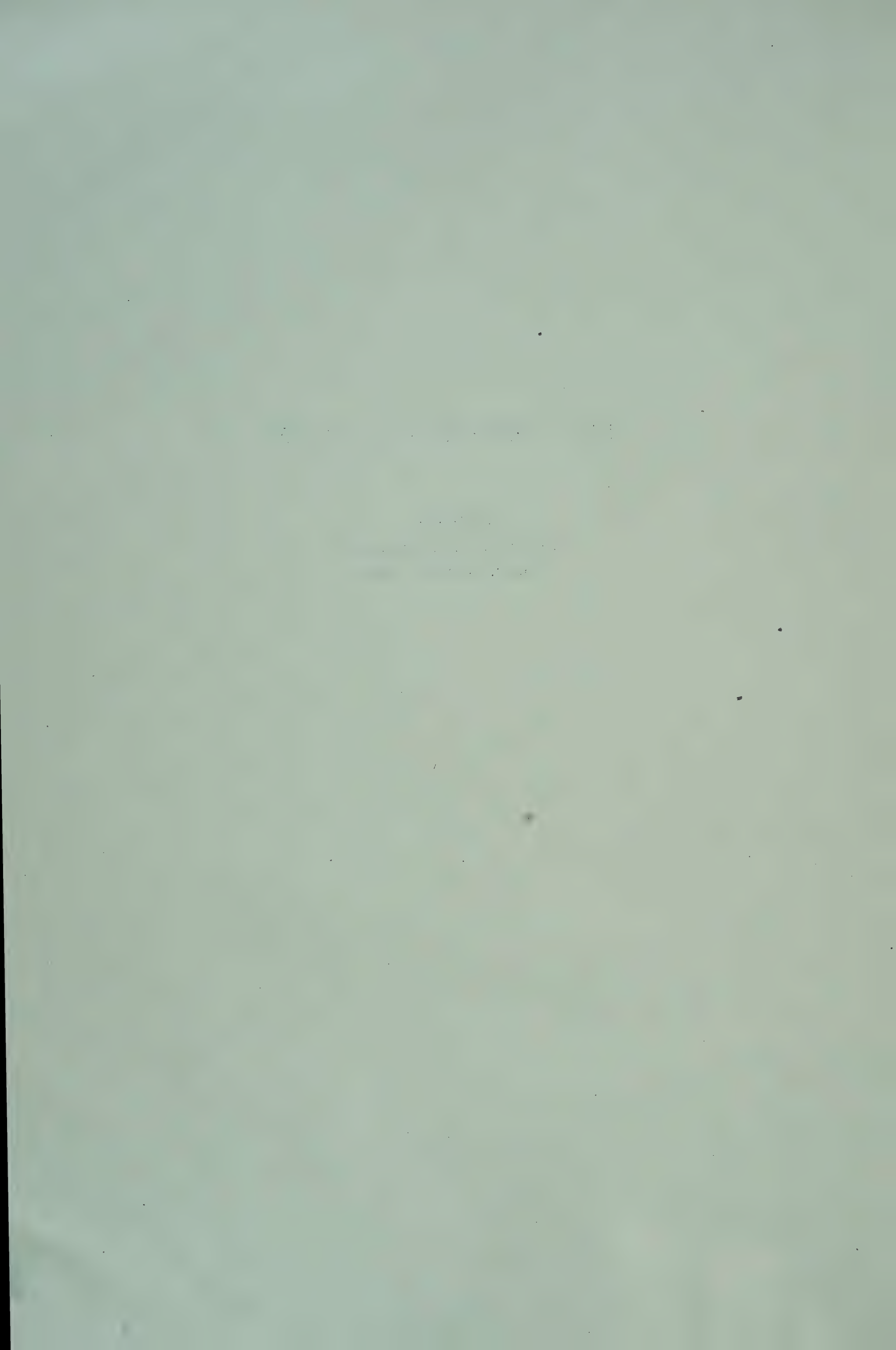
### III. TECHNOLOGY

*Moderators :*

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# LEAF PROTEIN TECHNOLOGY

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Leaf protein was first extracted in this laboratory from grass and water hyacinth in 1943 on a laboratory scale and fed to humans in the form of some preparations. But extraction of leaf protein on a large scale presents some special features as a problem in biochemical engineering, because one has to extract the protein economically from a large amount of cellulosic material without undergoing much change. A process developed in this laboratory involves the following unit operations.

*Crushing:* Crushing of the leaves is essential for bringing the protein into solution or suspension. This operation requires the highest amount of energy in the whole process. It has been found that the blade-type hammer mill can satisfactorily crush both tender and fibrous leaves and grass.

*Extraction:* Of the different extractants of protein used, *viz.*, water, ether-saturated water, aqueous sodium chloride, aqueous sodium carbonate and takadiastase<sup>1,2</sup>, 2 per cent sodium carbonate has been found to extract the material most from the leaves and grasses investigated. About 60 per cent of the protein is extracted by this reagent. The crushed mass is mixed with three times its weight of 2 per cent washing soda solution and stirred mechanically at room temperature for half an hour to effect its extraction.

*Separation of the fibres:* The extract is separated from the fibrous residue by centrifuging the slurry in a bucket centrifuge at about 500 r.p.m. The extract still contains some suspended leaf particles. It is filtered in a bag filter, when a clear green filtrate is obtained.

*Precipitation of the protein material:* The extract is fed into a steam-jacketed stainless steel heating pan. Commercial HCl is added with stirring to bring the pH to 3.8, which is the isoelectric pH of most of the leaf proteins. The proteins are precipitated. Steam is now turned on to raise the temperature to 80°C, when the precipitate is coagulated.

*Separation of the protein material:* The protein slurry is centrifuged in the bucket centrifuge fitted with canvas. The protein material forms a cake, which offers considerable resistance and the rate of flow of the filtrate gradually falls. The cake is removed, disintegrated in water and the slurry again centrifuged. This washing removes the adhering HCl, NaCl and some disagreeable odour and taste. The product obtained is yellowish-green.

*Drying of the product:* The protein cake can be dried in a tray drier with an air current at 60°C. Case-hardening occurs and the rate of drying is therefore slow. Besides, on drying, it becomes very hard and its pulverization becomes another problem. The drying operation becomes easier if the moist material is washed with a little alcohol. The dried product contains 50-60 per cent protein.

*Refining of the crude protein material:* The colour and odour of the crude product diminish its organoleptic quality. Extraction with methanol, rectified spirit, petroleum ether (40-60°) and acetone was tried to improve their quality. The last proved to be



the best and the traces of acetone were removed by washing with rectified spirit and the material was dried. The product was odourless and tasteless and had a faint colour,—yellowish in case of *Gliricidia*, chocolate in case of water hyacinth and greyish in case of lucerne, *Sesbania*, drumstick, arhar (*Cajanus cajan*) and *dub* grass. The protein content increased to 60-70 per cent. Since, however, rectified spirit is the cheapest among the solvents tried and is fairly easily recoverable, it would perhaps be the most suitable solvent under Indian conditions. Apart from improving the acceptability of the product, solvent extraction may improve its biological value and digestibility, as observed by the Japanese workers<sup>3</sup> working on *Chlorella*, who used methanol as the extractant.

Solvent extraction, however, is costly, though the cost can be reduced by recovering and recycling the solvent.

To reduce the cost of production, the by-products should be utilised. The fibrous residue containing about 30 per cent protein should be useful as fodder. The bulky filtrate remaining after the precipitation of the protein is rich in amino acids, sugars, minerals etc., and may perhaps be used as a fermentation medium. It has been found to be a good substitute for a 'casein hydrolysate-glucose-beef extract-potato extract' medium required for the production of mycobacillin, a new antifungal antibiotic.

The study of a wide variety of leaves and grasses has shown that the leaves of leguminous plants contain 30-40 per cent protein on dry weight basis and are easy to process. Maize leaves, sugarcane leaves and grasses are fibrous and difficult to process.

The proteins investigated show a high quality from amino acid analysis except for methionine.

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#### Discussion

- Q. Leaf and grass proteins are generally deficient in methionine. Some are also deficient in lysine. Would it, therefore, be desirable to concentrate on leaf proteins as supplements to cereal diets?
- A. Leaf proteins vary considerably with respect to their content of lysine and methionine, and proper choice of variety and blends have to be made for processing to extract proteins.
- Q. How does the amino acid content of leaf proteins vary in relation to variety, maturity and environmental factors?
- A. Not much work has been done on these aspects which deserve to be carefully investigated. It is, however, known that young leaves are richer in protein content. Therefore, high yielding forage crops have to be grown for the exclusive purpose of extracting proteins. The choice of raw material is the most crucial question determining further expansion and development of leaf protein production on a large scale.



- Q. Has the process indicated been worked on a pilot plant scale?
- A. Yes. A pilot plant has been put up in the Department of Food Technology at Jadavpur University, Calcutta.
- Q. What are the economics of the process?
- A. The cost of production of leaf protein would be the same as for protein isolates from other vegetable sources, such as groundnut.
- Q. How acceptable are the products?
- A. The isolates prepared have vegetable odour and green colour. They are acceptable to vegetarians as added to soups, chutneys or curries. They can also be used in admixture with cereal flours. For other uses the protein can be decolourised by solvent extraction which would of course add to the cost of the product.
- Q. Is there any use for the residues left after protein extraction?
- A. The residue after protein extraction contains enough nitrogenous and other nutrients. It can be washed free of alkali and used as cattle feed. The mother liquors from protein precipitation have been found to be good nutrient media for the growth of microorganisms for the production of certain antibiotics.



## PRODUCTION OF PROTEIN ISOLATE FROM GROUNDNUT (PEANUT)

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Oilseeds constitute some of the most concentrated forms of food in nature and contain fairly large quantities of protein besides the oil. Among the different constituents of the seed, only the oil has so far been considered of value for human consumption and the oil cake residues left behind after extraction have been used mainly as live-stock feed and manure. Scientific research conducted during the past few decades in this country and elsewhere has shown that the oilseed meal, if properly prepared and processed, can serve as a valuable protein supplement to human dietaries and that the proteins isolated from oilseed meals are likely to find extensive use in specialised food preparations suitable for feeding infants and children<sup>1</sup>.

Isolated vegetable proteins have certain advantages over the parent raw materials in that they are free from: (1) insoluble and indigestible carbohydrates which may swell and interfere in the digestion and utilization of protein particularly in children, (2) odoriferous and bitter principles present in the natural materials which may affect their palatability and (3) trypsin inhibitors, phytates etc., which may affect adversely their nutritive value. Further, protein isolates are 2-4 times as concentrated as the protein source and possess a bland taste, thus permitting ready blending with other natural foodstuffs for increasing their protein content without affecting their palatability.

### Processing of screw press groundnut cake for protein isolation

As a source of raw material for the commercial production of groundnut protein, the solvent extracted meal would be the natural choice as this could be processed to yield a fat free protein. Work carried out at the Institute had shown<sup>2</sup> that the screw press cake containing about 9 per cent fat could also be processed to yield a fat-free protein. The method consisted in the removal of fat from the cake dispersion by centrifugal separator thus eliminating the use of solvent extraction in the process. The process was, therefore, studied in greater detail and standardised<sup>3</sup>. The process, is briefly, described below:

Raw groundnuts are mildly roasted and decuticled. (Removal of cuticle has been found to be the most effective method of eliminating colour in the groundnut cake and the resulting protein isolate). The decuticled kernels are then pressed in screw-press, yielding a cake containing about 8.5 per cent N and 8-10 per cent fat. The cake is ground to a mesh size of 20-42 (ASTM) and then dispersed in water, keeping the meal to water ratio 1:15. The dispersion is adjusted to the pH range 8.0-8.5; after a peptization period of one hour, it is screened and clarified to obtain the carbohydrate fraction. The clarified protein solution is passed through a cream separator and most of the oil is obtained as a fat-rich emulsion. The protein is precipitated at pH 4.5 from the skimmed solution, the



precipitated protein curd washed, centrifuged and dried at 50°C in hot air. The flow-sheet of the process and yield data are given in Fig. 1.

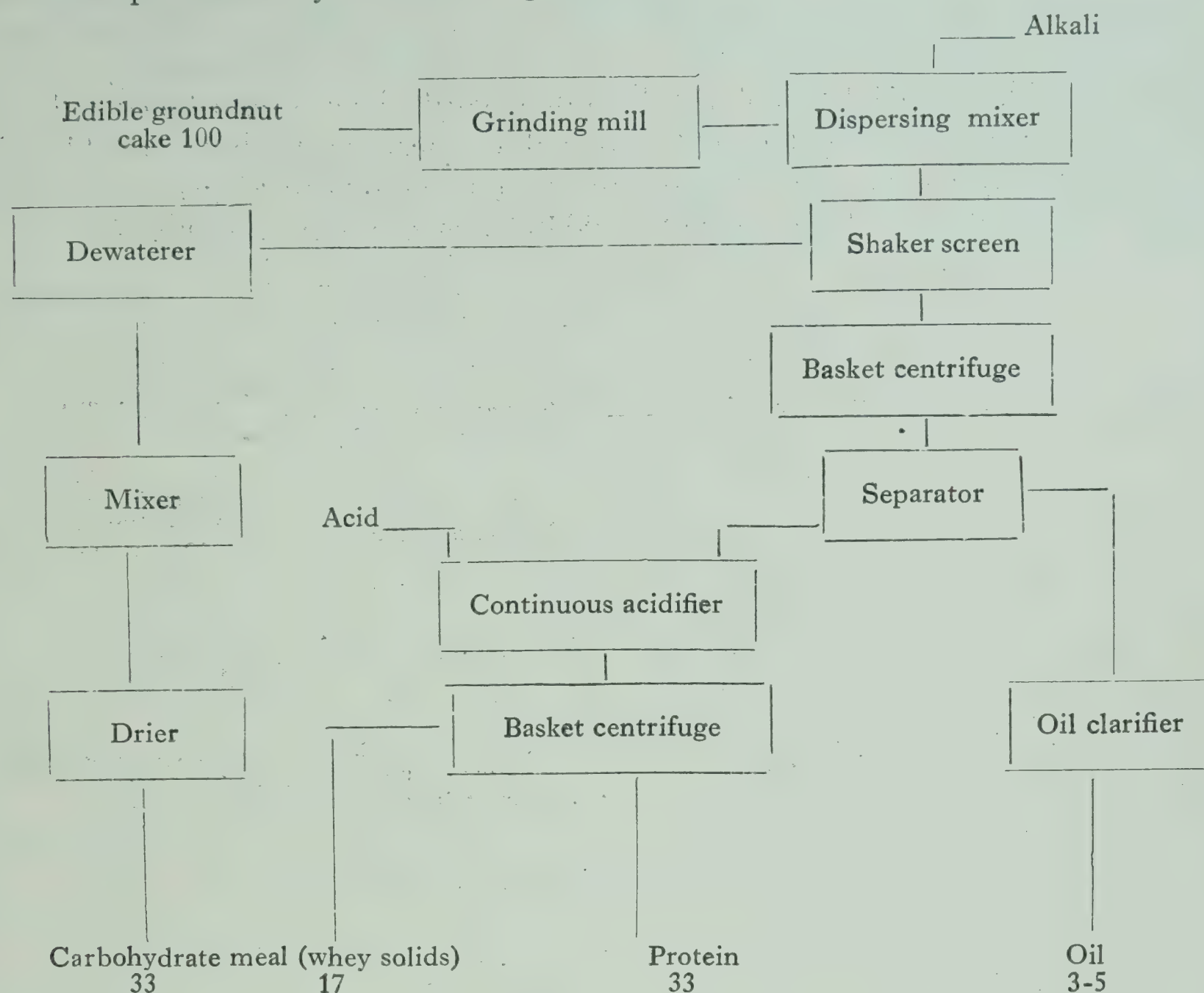


Fig. 1. Processing of screw-press groundnut cake

From the flow-sheet, two points emerge: (a) If the fat present in the press meal could be recovered by centrifugation, groundnut kernel itself may be used as raw material and its fat content recovered centrifugally (b) and the yield of protein may be improved by processing the kernel itself.

### Integrated processing of groundnut kernel for the separation of oil, protein and carbohydrate meal

When groundnut is dispersed in aqueous medium at optimum pH, the three major constituents namely, oil, protein and starch-fibre tend to separate from one another because of differences in specific gravity. This basic principle has been made use of in the development of the integrated method of processing groundnut which enables the simultaneous separation of oil, protein and the carbohydrate fractions.

The process, as originally developed, is described as follows: Decuticled (blanched) groundnut kernels are made into a paste and the paste is subjected to 'Skipin' process which consists in adjusting the moisture content of the paste to about 16-20 per cent at 35-37°C. About 30 per cent free oil on the weight of the paste which is



liberated at this stage, is drained off and the residual paste made into a dispersion in 8 volumes of water at pH 10.0. The dispersion is clarified to get a carbohydrate meal. The clarified dispersion is passed through a separating centrifuge to obtain another lot of oil equivalent to 12 per cent on the weight of the paste. The remaining skimmed dispersion is acidified to pH 4.5 to obtain the protein curd which is centrifuged and dried. The percentage yields of oil, protein and carbohydrate meal, on dry basis, are 42.0, 21.9 and 15.7 respectively, the oil recovery from the seed amounting to 85 per cent. A flow sheet of the process along with yield data for 100 lb batch size is given in Fig. 2.

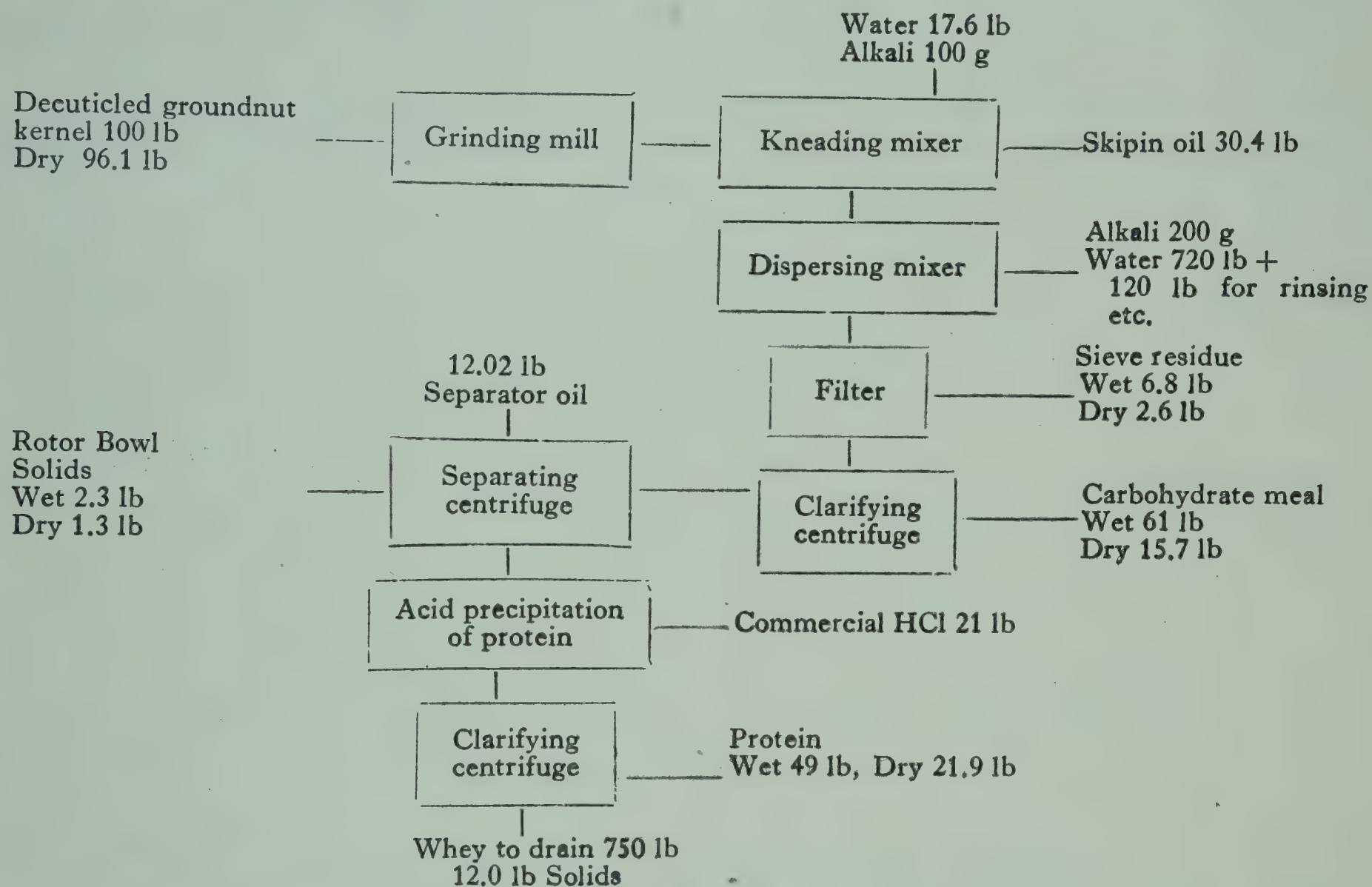


Fig. 2. Processing of groundnut kernel (original process)

The process worked with 100 lb batches of the kernel presented the following problems in scaling up:

- The dispersion prepared from the residue after the 'Skipin' process was prone to emulsification rendering its handling difficult. The very first step of clarification itself considerably emulsified the oil making clarification slow and incomplete, and the subsequent fat separation very inefficient. The rate of separation of the different constituents was too slow for the process to be feasible on a large scale.
- The 'Skipin' process, necessary as a preliminary step to reduce the fat content of the final emulsion, was a batch process, requiring skilled labour for operation. It also produced some degree of emulsification of the oil.

Consequently, the fat content of the protein isolate could not be reduced to below 10 per cent. Further improvements in the process were, therefore, effected.



## Modified Process

The extraction of flaked kernel with alkaline water brings into dispersion most of the protein, about 2/3 of the oil and some starch. The dispersion could be easily handled in continuous nozzle-type centrifuges to separate the protein solution, oil and the fine starch. The protein solution is then adjusted to pH 4.5-5.1 and the precipitated protein sedimented and dried. The unextracted residue after dewatering in a screw-press is dried and solvent-extracted to recover the remaining oil. The modified process yields a protein of relatively low fat (4 per cent) content and also makes possible the recovery of more oil (91-94 per cent), all of it in a refined state (less than 0.08 FFA). Our recent trials based on an understanding of the factors influencing emulsification of fat globules in the dispersion have indicated the possibility of further reducing the residual fat in the isolate. The flow-sheet of the modified process and yield data are given in Fig. 3.

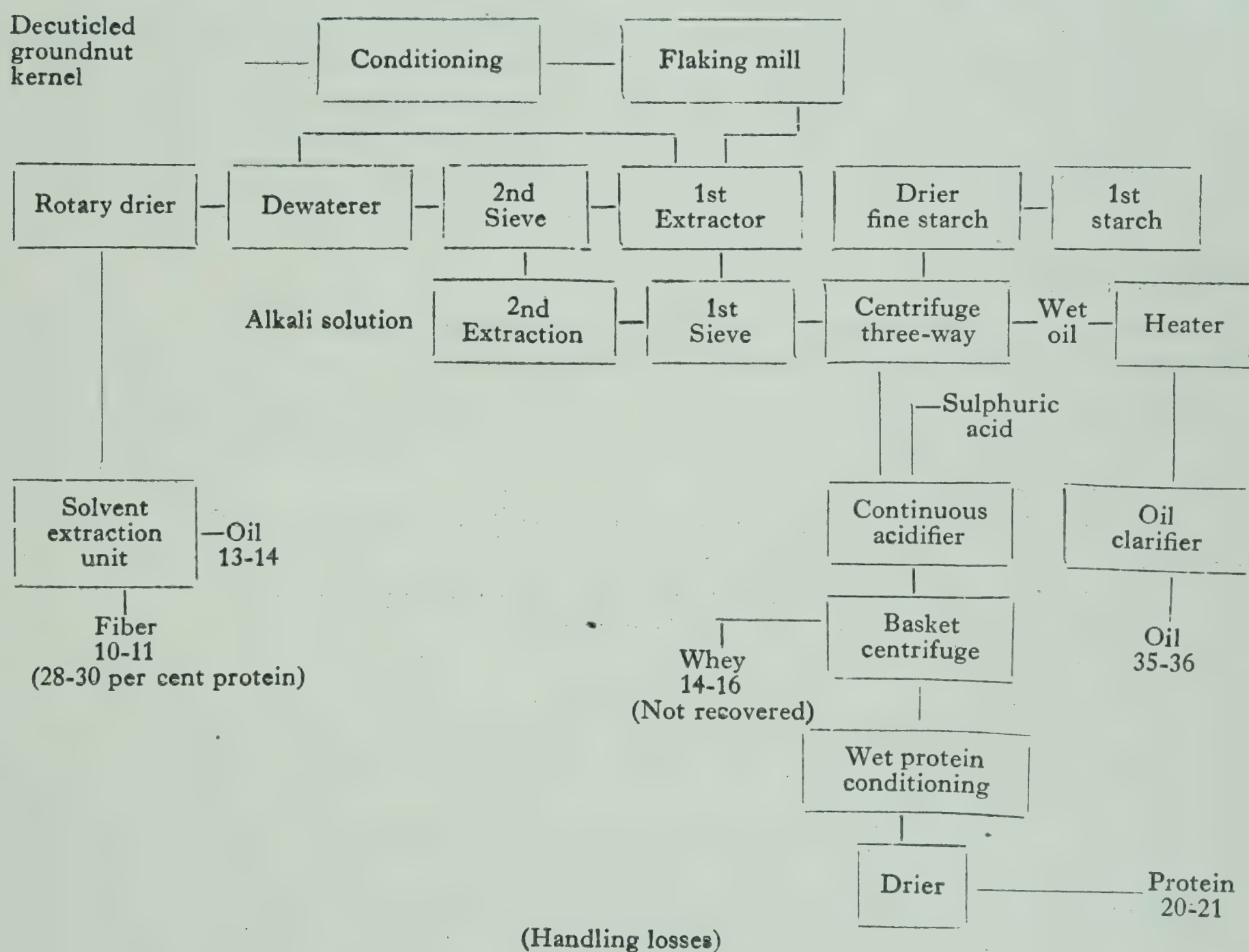


Fig. 3. Processing of groundnut kernel (modified process)

The process has the following important features:

(1) All the operations are capable of being carried out on a continuous basis, thus reducing considerably the time lag between the liberation of protein from the kernel and its being dried as an isolate.



(2) The oil obtained is in the refined state.

(3) The rate of separation of the different constituents is high enough to make the process feasible on a large scale.

Comparative analyses of the protein isolates obtained by the different procedures are given in Table I.

TABLE I  
*Comparative analyses of groundnut protein obtained from kernel and expeller cake*

Constituents		Protein from groundnut kernel by integrated process		Protein from expeller groundnut cake, after centrifugal separation of oil %
		Original %	Modified %	
Moisture	...	6.2	4.7	5.7
Fat	...	9.0	4.2	0.9
Ash	...	0.4	1.1	0.6
Protein (N $\times$ 6.25)	...	85.0	90.0	93.5

### Work in progress

Plans are being worked out for setting up a pilot plant with a capacity of 1000 lb of groundnut kernel per hour. This scale of operation would give adequate data to work out the commercial feasibility of the process. Studies are also in progress with regard to the evaluation of the nutritional quality of protein isolate and development of various formulations of protein-rich foods.

The 'wet processing' technique discussed above is being extended to other oil-bearing materials such as cottonseed, sesame, soyabean and coconut. The ultimate object is to make available for human consumption blended compositions based on different vegetable protein isolates.

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### Discussion

Q. What would be the cost of production of the peanut protein isolate?

A. It is estimated to cost 80 nP (16 American cents) per pound. The cost is inversely related to the scale of production. The cost would also decrease with improvements in processing that may be effected on the basis of experience during large scale production.



- Q. What is the temperature of extraction?
- A. The extraction is carried out at room temperature.
- Q. How does the oil obtained in this process compare with commercial groundnut oil?
- A. Since the oil is separated at low temperature and in alkaline medium a refined oil with very good keeping quality is obtained.
- Q. Could the process be applied to other oil seeds such as cotton seed, coconut, etc.?
- A. The optimal processing conditions have to be worked out in individual cases but the general principle of wet-rendering should be applicable in all cases.

*Additional comments by authors*

The Food Division of the Tata Industries, Bombay, is particularly interested in the exploitation of the wet-rendering process. With the assistance of the National Research Development Corporation and the co-operation of the Tata Industries, a commercial scale plant with capacity for processing 1,000 lb. of protein per hour or production of about 2 tons protein per day is proposed to be set up very soon.



# STUDY OF SOME OF THE OPERATIONS IN THE INTEGRATED PROCESS FOR GROUNDNUTS

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An integrated process for groundnuts, developed at this Institute is in some respects analogous to the wet-milling of corn. It aims at the separation of the fat, protein, starch, fiber and water-soluble constituents in a single process. Unlike corn, the groundnut kernel has a high content of fat distributed throughout the kernel which calls for some important variations in the process.

When groundnut is dispersed in water at an alkaline pH, the protein and the water-soluble constituents dissolve, leaving the fat and the insoluble carbohydrates in suspension. By centrifugation the fat is floated away and the carbohydrates sedimented, leaving the protein in the aqueous solution. The protein precipitates out on adjusting the solution to its isoelectric pH, and is sedimented. The water soluble constituents are carried away in the 'whey'. This is the basis of the integrated process<sup>1</sup>. Separation of fat by centrifugation and recovery of protein from the solution, being the two key operations of the process, have been studied in greater detail.

## Separation of fat

The separation of fat is dependent on the size of the fat globules in the dispersion. It was therefore considered necessary to study the effect of different operations on the size of fat globules, to avoid those operations that break up the fat globules to small size. The number of fat globules smaller than 2.5 microns diam. was taken as an index of the degree of emulsification caused by the different operations.

The count of fat globules was carried out by centrifuging the sample in a test tube for 5 minutes at 2,250 r.p.m. One ml of the central layer was pipetted out, diluted 50 times with distilled water and adjusted to pH 10. Fat globules, 2.5 microns or less, were counted on five diagonal squares of a haemocytometer, using a microscope ( $\times 500$  magnification). Counts for ten drops were taken for comparison with similar counts on the control. The ratio of the average count of sample to that of the control was expressed as the emulsification ratio.

The following operations were studied: (1) dispersion of the groundnut paste for varying times and at different pH values, (2) comparison of dispersions made from paste subjected to Skipin process<sup>3</sup> with dispersions made directly from the paste, (3) the effect of pumping using centrifugal diaphragm and paddle type pumps, (4) and various types of centrifuges were compared for the degree of fat emulsification produced during the operation of sedimentation of the carbohydrate fraction.

Preliminary experiments revealed that a cage type mixer was preferable to high speed propeller type stirrers. The pH of the dispersion and the time of dispersion had no significant effect on the fat globules. The emulsification produced by the different operations studied is shown in Table I.



TABLE I

Operation	Emulsification ratio	Remarks
Skipin ...	2.7	Planetary type mixer used
<i>Pumping</i>		
Centrifugal pump ...	1.1	Throttling on suction side
Diaphragm pump ...	1.0	
Paddle pump ...	1.5	
<i>Sedimentation</i>		
Sharples super centrifuge ...	3.0	Hollow bowl, 13,000xG
Super-D-Canter ...	5.7	Conical solid bowl, 3,000xG
ATM suspended solid basket ...	4.0	1,800xG
Escher Wyss horizontal basket ...	2.9	900xG
Westphalia laboratory ...	3.3	10,000xG

In the Skipin process<sup>2</sup> water is kneaded into the groundnut paste and produced an emulsification ratio of 2.7. The pumps caused no significant emulsification. The process of removing the carbohydrate fraction by sedimentation in a solid-bowl centrifuge produced maximum emulsification in the operations studied. It is, therefore, very desirable to avoid all operations that cause emulsification, before the separation of fat.

### Separation of protein

In the recovery of protein, the process of drying affected it more than any other operation. The following aspects of the recovery of protein were studied: (1) precipitation of the protein in a form holding minimum amount of water to reduce drying load, (2) determination of the highest temperature the protein could stand in the wet and dry states, and (3) different methods of drying.

The conditions of protein precipitation are important because they determine the yield and the moisture carried by it. The latter also depends on the efficiency of centrifugation.

The protein was precipitated at pH values between 4.0 and 6.3 and removed by centrifuging in two different types of centrifuges. The moisture content of the sedimented protein and the nitrogen content of the 'whey' were determined to find the optimum conditions of precipitation. The results are shown in Fig. 1. The protein was sedimented with the minimum quantity of moisture, when precipitated at pH 5.1, in both types of centrifuges. The loss of protein in the 'whey' was minimum in the range 4.7-6.0. It is therefore concluded that the yield of the protein will be maximum in this range.

The wet protein obtained by centrifuging was subjected to various temperatures (40, 50, 60, 70, 80 and 90°C ± 1) for 1 hour in a closed dish. Samples were spread in thin layers and the dish closed to prevent drying, and later freeze-dried. A sample of protein was freeze-dried directly to serve as a control reference. For study of the dry-heat treatment, the wet protein was freeze-dried and the dry samples maintained at temperatures of 80, 90, 100, 105, 110 and 130°C for three hours. A longer period was used for the dry material as the removal of water in the later stages is slow in the drying process.



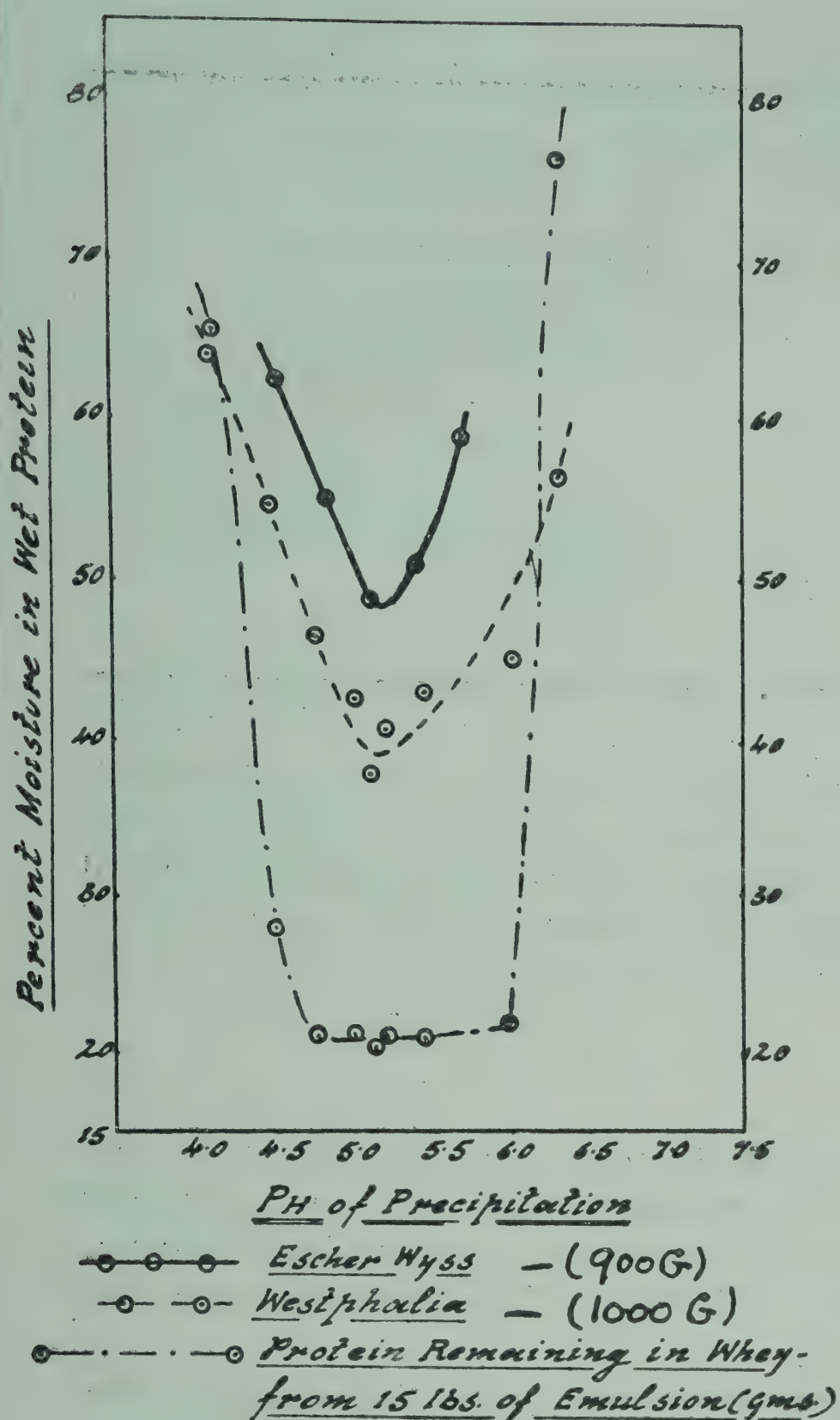


Fig. 1.

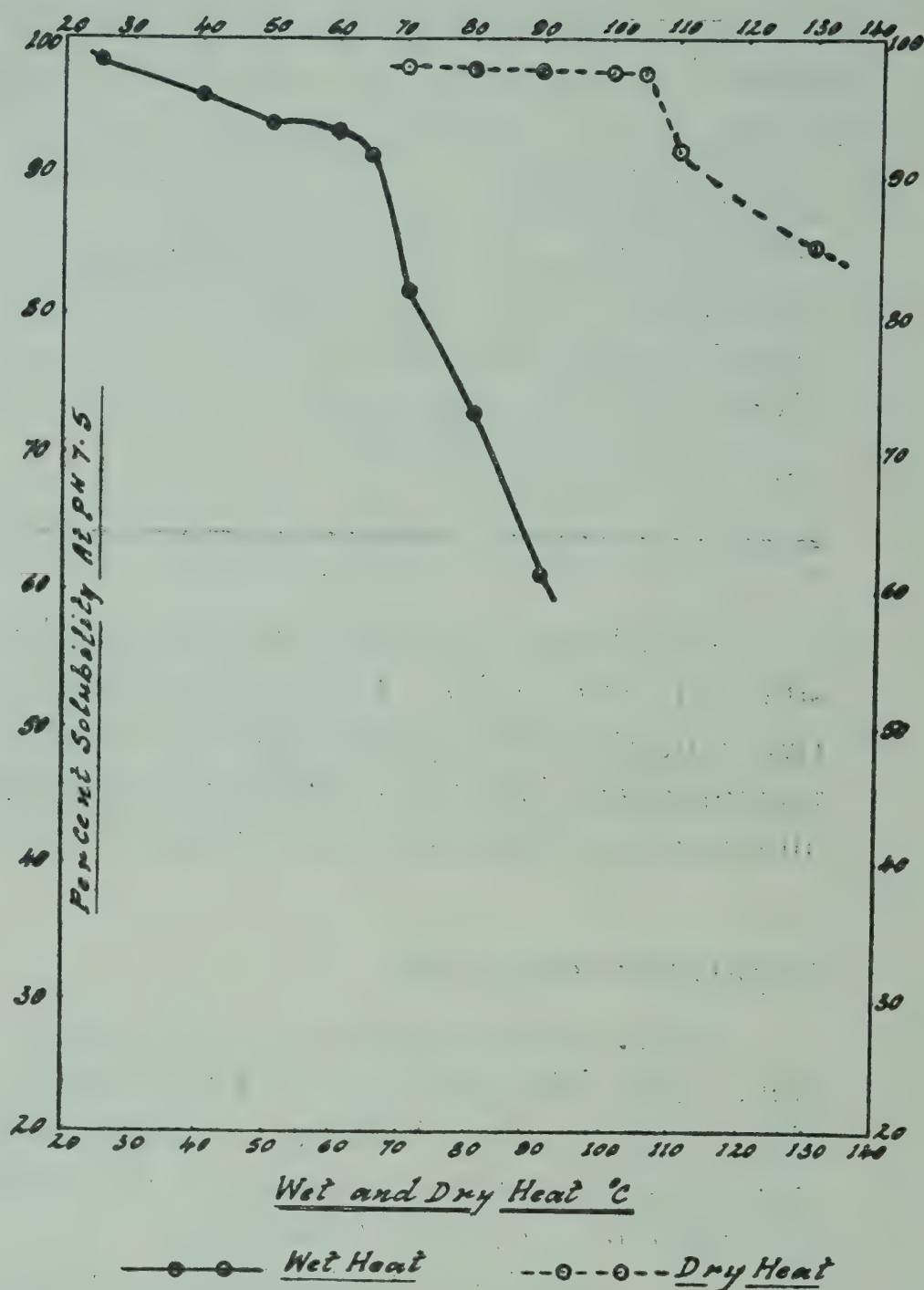


Fig. 2

The solubilities in water at pH 7.5, of the variously treated samples of protein and the controls, are shown in Fig. 2. It will be seen that a rapid decrease in solubility is caused at temperatures greater than 60°C, when the wet protein is subjected to heat. In the dry state, however, even a temperature of 100°C for three hours does not cause any drastic reduction in solubility. It may, therefore, be concluded from the results that if the wet protein is to be dried in a stream of air, the wet bulb temperature of the air may be raised up to 60°C in the constant rate period, while in the falling rate period the dry bulb temperature may be raised up to about 100°C, without affecting the solubility of the protein to any appreciable extent.

The precipitated protein is capable of being dried in different ways, such as in a truck drier, drum drier, or a spray drier or under vacuum in a shelf drier. Each method has its own advantages and disadvantages. Depending on the end uses of the product one or the other method of drying could be preferred. To select the proper drier, the nature of the



product produced by each type of drier must be known. Protein prepared under mild conditions was dried by the different methods and the solubility in water and bulk density measured. The results are given in Table II. The control sample was freeze-dried and had a solubility very close to 100 per cent. The spray drier and the drum drier have the advantages of simple continuous operation that can be easily linked up with any continuous process for the precipitation and sedimentation of protein. Experiments to modify the air-drying process to suit continuous production are under way.

TABLE II

Method of drying	Solubility* %	Bulk density g/ml
Drum drying (pH 7.3) ...	65.0	0.1
Truck drier (pH 5.0) ...	92.5	0.7
Vacuum shelf (pH 5.0) ...	95.8	0.7
Vacuum shelf (pH 7.2) ...	95.5	0.6
Spray dried (pH 6.8) ...	98.0	0.3

\* In water at pH 7.5.

## Summary

Emulsification produced by various operations, such as the Skipin process, pumping, centrifugal sedimentation of the carbohydrate fraction etc., on groundnut dispersion in water have been studied. Most of the centrifuges used for the clarification step were found to produce emulsification. Moisture content of protein precipitated at various pH, was determined; pH 5.1 is optimum for minimum moisture with maximum yield of protein. The sedimented protein is stable up to 1 hour at 60°C in the wet state or up to 100°C for 3 hours in the dry state. Among the different methods of drying only drum drying reduces the solubility to a considerable extent.

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# TECHNOLOGY OF PROTEIN-RICH NUTRO FOODS

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According to FAO Food Balance Sheets<sup>1</sup> the average daily *per capita* protein consumption in India is 50.4 g as against the usually recommended level of 60 to 70 g. The potentialities of augmenting the protein intake of the people from untapped edible oil-seed meals are, indeed, great when it is realised that India produces over 7.29 million tons of oil seeds of which groundnut kernels alone account for 2.87 million tons<sup>1</sup>.

Pioneering investigations carried out at the Central Food Technological Research Institute have led to the establishment of a pilot plant for the production of an acceptable quality of groundnut flour containing 45-50 per cent protein and 5-10 per cent residual oil. Apart from adopting this flour as the major component of Indian Multipurpose Food<sup>2,3</sup> and as the chief source of protein in the Tapioca Macaroni products<sup>4,5</sup> various other palatable protein-rich foods designated as 'Nutro Foods' have also been developed (Table I). These products comprising Nutro Biscuits, Nutro Macaroni, Nutro Breakfast and Nutro Protein Food for infants and other age groups have all been fortified with calcium and vitamins, the deficiencies of which are widely prevalent in our country. This paper deals with the pilot plant production, shelf-life studies and nutritive value of these

TABLE I  
*Composition of Nutro protein rich foods*

	Biscuits	Wheat macaroni %	(Formulation A) Tapioca macaroni %	Breakfast food porridge <sup>2</sup> %
Moisture ...	2.0	11.7	9.8	3.4
Protein (N × 6.25) ...	16.5	19.3	18.0	40.4
Fat <sup>1</sup> ...	17.7	2.5	2.1	7.4
Ash ...	2.3	1.26	2.83	3.5
Calcium as Ca ...	0.22	0.39	0.49	0.06
Phosphorus as P ...	0.20	0.17	0.45	0.36
Iron, mg ...	1.20	1.40	3.20	...
Thiamine, mg ...	1.10	0.59	0.70	0.70
Riboflavin, mg ...	2.53	1.04	0.70	0.19
Nicotinic acid, mg ...	34.30	7.60	5.6	13.80
Vitamin A, I.U. ...	1,520	...	1,500	...
Vitamin D, I.U. ...	700	...	100	...

<sup>1</sup> By acid hydrolysis.

<sup>2</sup> Not enriched.



products. Consumer acceptability tests have shown these foods to be palatable and they can, therefore, be produced on a commercial scale.

1. **Nutro biscuits:** These are sweet biscuits which are very popular in India. But, unlike the customary sweet types, they contain over 16 per cent protein and a full complement of vitamins—A, D, B<sub>1</sub>, riboflavin, nicotinic acid and calcium. The high level of protein was achieved by replacing wheat flour in the formulation with groundnut flour to the extent of 40 per cent. Typical batch composition adopted for mechanised production in a modern biscuit factory<sup>6</sup> is shown in Table II.

TABLE II  
*Typical batch composition for Nutro biscuits*

<i>Main ingredients</i>				
Wheat flour (Maida), lb	...	...	...	175
Groundnut flour, lb	...	...	...	112
Sugar, lb	...	...	...	80
Shortening, lb	...	...	...	68
Glucose syrup, lb	...	...	...	4
Calcium carbonate (B.P. Light), lb	...	...	...	3
Common salt, oz.	...	...	...	30
<i>Vitamins</i>				
Thiamine hydrochloride (B <sub>1</sub> ), g	...	...	...	4.5
Riboflavin (B <sub>2</sub> ), g	...	...	...	3.5
Nicotinic acid, g	...	...	...	35.0
Vitamin A (as acetate or palmitate) Million I.U.	...	...	...	13.5
Vitamin D (as calciferol), mg	...	...	...	40.0
<i>Baking chemicals</i>				
Ammonium bicarbonate, lb	...	...	...	6-7
Sodium bicarbonate, oz.	...	...	...	28
Rex baking powder, oz.	...	...	...	28
Water, lb	...	...	...	35
<i>Flavours</i>				
Raspberry, oz.	...	...	...	4
Orange oil, oz.	...	...	...	2
Vanillin, oz.	...	...	...	6-7
Antioxidant 'Sustaine' (butylated hydroxyanisole containing citric acid and propyl gallate, by wt of fat used)	...	...	...	0.02

The granularity of groundnut flour was found to influence the properties of dough and texture of the biscuits. If it is as fine as wheat flour (IOXX), water absorption increases, the dough becomes sticky and the biscuits are hard and tend to puff during baking. Less sticky dough and better biscuits were obtained by using slightly coarser flour (about 66 per cent retained on the 100 mesh sieve).

**Preparation:** Homogeneously blended dry ingredients are mixed with creamed shortening containing the antioxidant, vitamin A, D, glucose syrup, ammonium bicarbonate, sodium bicarbonate and adequate amount of water. Either the rotary embossing machine or the sheeting and punching machines can be used to make biscuits which are baked in a continuous oven at 450-500°F for 5-6 minutes, cooled and packed.

**Consumer acceptance:** In 1957, the Prime Minister of India donated one lakh of rupees towards the purchase of 1,00,000 lb of Nutro biscuits, which offered a unique opportunity



TABLE III

*Average values of the initial and final measurements of children in the control and experimental groups  
(18 children per group)*

Charter	Control group (Rice diet)			Experimental group (Receiving Nutro macaroni)			Difference in increase (experimental minus control)
	Initial	Final	Increase	Increase	Initial	Final	
Height (Inches) ...	51.02	51.81	0.79	51.07	52.05	0.98	$0.19 \pm 0.14^*$
Weight (Pounds) ...	50.33	54.37	4.04	49.99	56.70	6.71	$2.67 \pm 0.76^\dagger$
Haemoglobin (g/100cc) ...	12.16	11.81	-0.35	11.84	12.32	0.48	$0.83 \pm 0.83^\ddagger$
Red blood cell count (mil/cu.mm) ...	4.45	4.38	-0.07	4.38	4.46	0.08	$0.15 \pm 0.10$

\* Standard error of the mean based on 17 degrees of freedom.

† Significant at 1 per cent level.

‡ Significant at 0.1 per cent level.

to organise production on a large scale. About 50 tons of Nutro biscuits were manufactured for distribution by the Indian Red Cross Society, in consultation with the Prime Minister's Relief Fund Organisation, to a number of hospitals, children's schools, welfare centres, earthquake affected areas, and the State Branches of the Indian Red Cross Society. The popularity of the biscuits induced a commercial firm to take up production.

**Nutritive value and shelf-life:** While developing the formulation, necessary precautions were taken to ensure good shelf-life. This has been confirmed by storage tests<sup>7</sup>. The biscuits have shown good shelf-life and the over-all nutritive value determined by the growth method has been found satisfactory<sup>8</sup>.

**2. Nutro macaroni:** Macaroni products in different shapes and sizes are normally made from durum wheat semolina. Use of eggs, milk, gluten<sup>9</sup>, fish<sup>10</sup>, and soyabean<sup>11,12</sup> in certain proportions along with wheat semolina has been reported. Nutro macaroni is based on a blend of wheat semolina (80 per cent) and low fat groundnut flour (20 per cent), with extra fortification with calcium, as calcium carbonate (0.9 per cent), 0.5 mg thiamine hydrochloride, 1.0 mg nicotinic acid, 1.0 mg riboflavin, and 135 I.U. of vitamin D as calciferol.

**Pilot plant studies:** The product has been made in different shapes, *viz.*, shells, rice, tubes etc., in large quantities in our Macaroni Pilot Plant<sup>4</sup>. The technique of manufacture is basically similar to that of wheat macaroni. The incorporation of groundnut flour and calcium carbonate was found to affect the consistency of the dough. The water absorption of the dough has to be controlled to ensure proper extrusion.

A study of the effect of processing and the mode of cooking on the nutritive value of the product<sup>13</sup> has shown that the added vitamins are fairly stable. The technique of cooking the product with an appropriate amount of water (1:4) avoids loss of nutrients in the gruel.

Institution feeding trials on 36 subjects, aged 4-12 years, over a period of 24 weeks, were conducted to assess the effect of replacing 50 per cent of rice in their diet by Nutro macaroni<sup>14</sup>. The product was found to be acceptable, and statistically significant gains in weight (Table III) and improvement in nutritional status (Table IV) of children taking Nutro macaroni supplemented diet were recorded.



TABLE IV  
*Changes in the nutritional status of children in the control and experimental groups*

Group	Nutritional status		Deteriorated	Total
	Improved	Stationary		
No. of children control (rice diet) ...	4	14	...	18
Experimental (Nutro supplemented rice diet) ... ..	13	5	...	18

TABLE V  
*Batch composition of Nutro protein rich food (100 lb Batch)*

FORMULATION					
Ingredients				A lb	B lb
Groundnut flour	...	...		25.0	30.0
Wheat semolina	...	...		22.5	22.5
Tapioca flour	...	...		50.0	47.6
Casein	...	...	...	2.5	0.0
Fortification in each case					
Minerals			Vitamins		
Calcium carbonate	...	0.5 lb	Thiamine hydrochloride, mg	...	125
Tri calcium phosphate	...	0.5 lb	Riboflavin, mg	...	250
			Calcium pantothenate, mg	...	250
			Vitamin A, I.U.	...	7.5 lakh
			Vitamin D (Calciferol) I.U.	...	0.5 lakh

*Shelf-life:* Systematic storage tests<sup>15</sup> have established that the product has a good shelf-life. No deterioration in the organoleptic quality of the product was observed over a period of 12 months when stored in sealed polythene bags (250 gauge).

**3. Nutro protein food:** The existing proprietary brands of processed cereal foods for children are beyond the reach of a vast section of Indian families. Products based entirely on milk solids are available at rather high prices. The desirability of having a reasonably priced, nutritionally balanced protein-rich food, adequately fortified with vitamins and minerals, hardly needs any emphasis.

*Processing:* The technique developed for obtaining suitable dough for macaroni products<sup>4,16</sup> has been applied successfully for this product also. The ingredients (Table V) are blended thoroughly, prior to doughing with boiling water (30-35 parts). The dough, which is fairly stiff, is vacuum extruded (ATA press), through a die in the shape of 'ringlets'



or 'alphabets' which, after passage through a pre-dryer, are steamed for about ten minutes and subsequently dried in a Buhler dryer (Type TTHA<sup>4</sup>). Steaming, though a difficult step in the operation, nevertheless, imparts better flavour and cooking quality to the product.

*Nutritive value:* The product is being subjected to feeding tests with laboratory animals, and feeding tests on children are also in progress at two centres.

At 10 per cent level of protein intake, the protein efficiency ratio of formulation A and B has been found to be 1.73 and 1.47 respectively. The liver fats averaged 14.9 and 17.5 per cent respectively (on dry basis).

Growth promoting value of the product, when it formed the sole diet having 10 per cent groundnut oil as a supplement, has been determined by rat-growth method. Average weekly gains in weight of 14.3 g and 13.8 g with formulations A and B respectively have been obtained. Experiments to determine the extent to which the use of this product can economise the quantity of milk required for feeding children are in progress.

*Acceptability:* Laboratory culinary tests suggest that this type of product should find very wide acceptance for the following reasons:

- (1) It takes only 4-5 minutes for cooking.
- (2) The cooked product is more or less bland in taste; it can, therefore, be incorporated into a variety of Indian food preparations.
- (3) Consistent use of this product will ensure supply of much needed proteins, minerals and vitamins.
- (4) It can serve as a very good breakfast food, and when cooked with milk, it becomes very tasty.

**4. Nutro breakfast food:** This is a porridge-type product having a high protein content (42 per cent) and good flavour and taste. By processing a blend comprising 80 parts groundnut flour, 20 parts wheat flour, and 1-2 per cent malt extract, it has been possible to modify the characteristic taste of groundnut flour.

*Process:* The dry ingredients are made into a stiff dough, which is cooked under pressure followed by granulation, drying and slight toasting. The contribution of wheat component to flavour development seems specific, because the use of other cereals or starches does not bring about such a modification. Fortification of this product both with vitamins and minerals has to be studied.

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# STUDIES ON THE NUTRITIVE VALUE OF PROCESSED PROTEIN FOODS BASED ON OIL SEED MEALS FORTIFIED WITH VITAMINS AND MINERALS

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Large quantities of potential protein rich foods of vegetable origin, e.g., oil seed meals and pulses, are available in the country and by suitable processing and fortification with vitamins and minerals, it should be possible to prepare, on a large scale, low cost processed protein foods which can be used as supplements to the diets of low income groups of the population. The results of investigations carried out on the preparation and the nutritive value of processed protein foods based on oil seed meals and pulse flours are presented in this paper.

## Availability of raw materials

The protein rich foods of vegetable origin which are available in plenty in the country are the edible oil seed meals and pulses, e.g., low-fat groundnut meal, coconut meal, sesame meal and Bengal gram. Of the various oil seed meals, only groundnut meal is available in large quantities. Coconut meal is available in fairly large amounts in the Kerala state as a by-product of the coconut oil industry. The high fibre content of coconut meal, however, precludes its use as a sole protein supplement to the diet. The figures for the production of certain oil seeds and Bengal gram in the country<sup>1</sup> are given in Table I.

TABLE I  
*Annual production of certain oilseeds and Bengal gram*

Name of material	Production for 1954-55 (tons)
Groundnut in shell ...	3,823,000
Sesame seeds ...	592,000
Copra ...	185,000
Bengal gram (whole pulse) ...	5,125,000

## Requirements of processed vegetable proteins

In planning vegetable protein mixtures for supplementing human diets, the WHO protein Advisory Committee<sup>2</sup> suggested that it is necessary to take into consideration the following factors: (1) the amino acid content of the individual ingredients and the final products, (2) the possible presence of toxic or interfering factors, (3) the need for



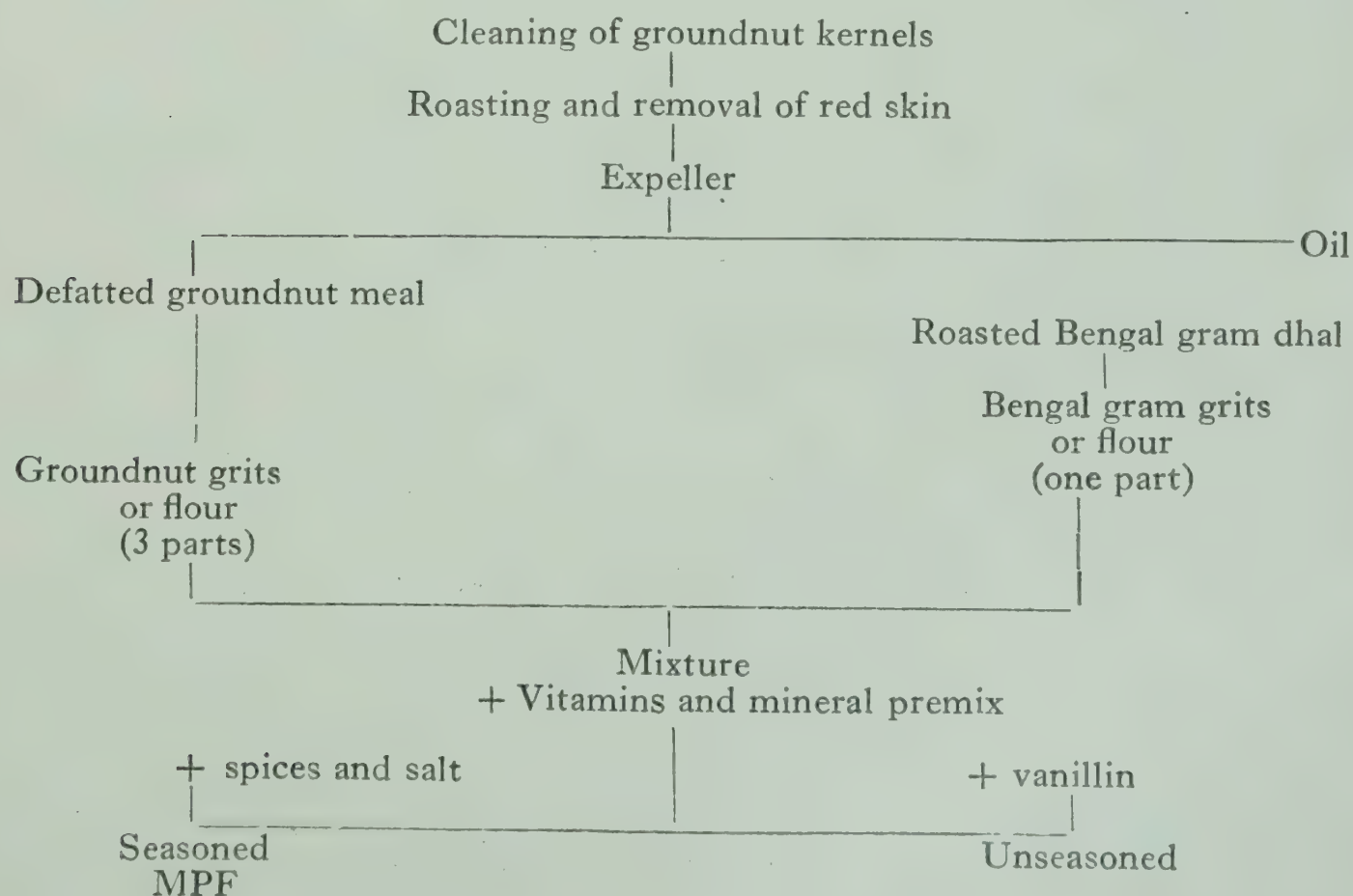
obtaining exact specifications for each of the components, (4) the necessity of avoiding processes that damage the quality of the protein, (5) the desirability of using products of local origin, (6) the low cost and good keeping quality of the product, (7) the suitability of the product for feeding weaned infants and (8) the acceptability of the product to the consumers. The products should not be recommended for commercial production until these are found satisfactory by the following tests: (1) freedom from toxicity as tested by animal experiments, (2) moderately high biological value of its proteins as assessed by animal growth studies, (3) demonstration of the supplementary value and acceptability of the product to the diet of children under careful medical supervision and (4) large scale feeding trials in selected population groups to assess its general acceptability. Although the primary objective is to provide a supplementary source of protein of good quality, it is desirable that a vegetable protein mixture, *should also contain adequate quantities of vitamins and minerals that are likely to be lacking in the diets of the low income groups of the population.* This can be achieved by fortification with minerals and synthetic vitamins which are available in large quantities at low cost.

### Preparation of low-cost protein foods

The groundnut flour and Bengal gram flour used in the preparation of the protein foods were prepared according to the procedure shown in Fig. 1. The low-fat coconut meal of edible quality was obtained from Messrs Tata Oil Mills, Ernakulam. Conditions were standardized for the production of two protein foods namely Indian Multipurpose Food (MPF) and a low-cost protein food containing coconut meal. Indian Multipurpose Food consisted of a blend of specially processed groundnut flour (75 parts) and Bengal gram flour (25 parts). The low-cost protein food contained a blend of low-fat coconut meal (25 parts), low-fat groundnut flour (50 parts) and Bengal gram flour (25 parts). Both

FIG. 1

*Flow sheet for the manufacture of Indian MPF*





the protein foods were fortified with vitamins A, D, thiamine and riboflavin and calcium as calcium phosphate. The flow sheet of the process for the manufacture of Indian Multipurpose Food is shown in Fig. 1.

The chemical composition of the protein foods as compared to skim milk powder and American Multipurpose Food which have been suggested as supplements to poor human dietaries is given in Table II. It is evident that a daily supplement of 2 oz. of the protein foods will provide a third of the daily requirements of protein, calcium, vitamin A and riboflavin for adults and nearly half the requirements for children.

TABLE II  
*Percentage chemical composition of the different protein rich foods*

		Indian Multipurpose food	Low-cost protein food	Skim milk powder	American Multipurpose food	Indian Multipurpose food (formula C)	Balanced Malt food
Protein (N × 6.25)	...	41.9	36.5	35.0	42.3	41.4	31.8
Fat	...	8.5	7.5	1.0	7.6	6.4	4.9
Carbohydrate	...	35.8	39.4	53.1	36.9	38.9	48.0
Calcium (g)	...	0.665	0.480	1.3	0.587	0.791	0.58
Phosphorus (g)	...	0.820	0.650	1.03	0.440	0.856	0.55
Iron (mg)	...	5.1	8.7	0.6	7.0	5.1	6.7
Thiamine (mg)	...	1.3	1.4	0.35	0.7	1.9	1.5
Nicotinic acid (mg)	...	14.0	10.9	1.1	7.0	12.3	8.5
Riboflavin (mg)	...	3.0	3.1	1.39	1.2	1.8	3.0
Vitamin A (I.U.)	...	3,000	3,000	...	2,940	3,000	3,000
„ D (I.U.)	...	300	300	...	23.5	300	300
Calorific value	...	387	372	361	386	379	364

Studies on the nutritive value of the protein foods

The nutritive value of Indian Multipurpose Food and low-cost protein food containing coconut meal was determined by experiments on albino rats.

Overall nutritive value of Indian MPF

The overall nutritive value of Indian Multipurpose Food as compared with American Multipurpose Food was first determined. The protein foods constituted 40 per cent of the diet and were the sole sources of proteins, minerals, B-complex vitamins and vitamins A and D. Two groups of freshly weaned young rats (weighing between 40-50 g. and distributed equally according to sex, litter and body weight) were fed on the experimental diets for a period of eight weeks. The average weekly increase in weight of rats fed on the



TABLE III  
Average weekly growth of rats fed on the experimental diets  
(Duration of experiment: 8 weeks)

Diet	Average protein content of the diet (moisture free basis)%	Average initial body weight (g)	Average daily food intake (dry wt basis (g)	Average weekly gain in weight (g)
Indian MPF	18.64	47.0	11.60	13.8
American MPF	18.80	46.7	11.70	14.8

}  $\pm 0.35$   
(10 d.f.)

different diets is given in Table III. After feeding for a period of 8 weeks, haemoglobin and red blood cell count of all the experimental animals were determined from the blood drawn from the tail of rats. The rats were then sacrificed and analysis of liver and body for moisture, protein and fat was carried out. The results showed no significant difference in the composition of the liver or body of the experimental rats. The diet containing Indian Multipurpose Food also promoted satisfactory reproduction and lactation in rats.

### Supplementary value to poor Indian diets

Animal experiments were also carried out to study the supplementary value of the different protein foods to poor vegetarian diets based on different cereals and millets. The composition of the experimental diets was the same as that described by Subrahmanyam *et al.*<sup>6</sup> Groups of freshly weaned albino rats were fed *ad lib* on poor vegetarian diets based on rice, wheat, *jowar* and *ragi* and the similar diets in which 12.5 per cent of the cereal or millet was replaced by the protein foods. The data regarding the average weekly increase in body weight and the daily food intake of the animals are given in Table IV. The results on statistical analysis showed that both the protein foods when incorporated at 12.5 per cent level had a marked supplementary value to the poor diets based on different cereals.

TABLE IV  
Supplementary value of Indian MPF and low-cost protein food containing coconut meal to poor vegetarian diets

Diet	Average weekly increase in weight of rats (g)		
	Control	Supplemented with	
		Multipurpose food	Low-cost protein food
Rice	5.0	14.6	14.3
Wheat	7.8	12.5	13.8
Jowar	8.5	14.0	13.9
Ragi	8.4	13.3	13.2



## Nutritive value of the proteins

The biological value of the proteins of the foods was determined both by the nitrogen balance method of Mitchell<sup>3</sup> and rat growth method of Osborne, Mendel and Ferry<sup>4</sup>. The results (Table V) show that the proteins of the two foods are of fairly high biological value.

TABLE V  
*Nutritive value of the proteins of Indian MPF and low-cost protein food*

Protein food	Biological value	Protein efficiency ratio (8 weeks)
Indian MPF	59.5	1.40
Low-cost protein food	54.8	1.70

## Value of Indian Multipurpose Food in overcoming protein deficiency in rats

Investigations on albino rats were carried out to study the relative efficacy of Indian Multipurpose Food, Bengal gram and skim milk powder to meet the protein requirements of protein depleted rats. Three groups of protein depleted rats were fed on the experimental diets containing the different protein rich foods, which provided 14 per cent of protein in the diet for a period of three weeks. A metabolism study was carried out during the period to determine the retention of nitrogen in the rats fed on the different diets. The increase in the cell solids, which is a measure of the protein content, of the rats was also determined. The results indicate that the ability of the different proteins to meet the protein needs of protein depleted rats ranged in the following descending order: skim milk powder, Multipurpose food and Bengal gram.

## Supplementary value of Indian Multipurpose Food and low-cost protein food containing coconut meal to the diets of school children

Feeding trials were carried out in two local boarding homes to study the effect of a supplement of (2 oz per day) Indian M.P.F. or low cost protein food on the growth and nutritional status of school children. The subjects were girls and boys aged 4-12 years. They were examined clinically and only those found free from diseases likely to interfere with the experiment were included. Height, weight, nutritional status, red blood cell count and haemoglobin content of the blood were determined according to the method of Reddy *et al*<sup>5</sup>. On the basis of initial height and weight the children were paired and the members of each pair allotted at random to two groups.

A dietary survey, carried out according to Reddy *et al*<sup>5</sup>, revealed that the diets consumed normally by the children were deficient in calories, proteins, certain vitamins and calcium. Each subject in the experimental group was given a daily supplement of 2 oz of protein food: 1 oz in the form of *chutney* with lunch and 1 oz in the form of soup with dinner. In order to equalise the calorie intake in the two groups, each subject in the control group was given daily one ounce maize starch and one ounce sugar in the form of pudding.



The subjects were fed three times a day. Breakfast consisted of rice or a wheat preparation (*Uppumav*, home made bread, *chappati*, etc) and a glass of milk or coffee. Lunch and dinner consisted of rice and a vegetable soup preparation. The feeding was carried out for a period of 5-8 months, at the end of which the height, weight and nutritional status of the children was again determined.

The results (Tables VI-VIII) show that the improvement in height, weight and nutritional status of the children receiving the protein supplements daily are significantly greater than those observed in the control children.

TABLE VI  
*Increase in height, weight, haemoglobin and R.B.C. count of control and experimental children*  
(23 children in each group)  
(Period—5 months. Exp. gr. 2 oz of Indian MPF)

	Control (Rice diet)	Experimental (Rice-MPF diet)	Significance of difference
Height (inches) ...	0.52	0.96	Sig. at 1%
Weight (pounds) ...	1.00	2.61	„ „ 0.1%
Haemoglobin (g/100cc) ...	0.13	1.60	„ „ 5%
Red blood cell (106/cu. mm)	0.07	0.33	„ „ 1%

TABLE VII  
*Increase in height, haemoglobin and R.B.C. count of control and experimental children*  
(20 boys in each group)  
(Period—8 months. Exp. gr. 2 oz of low-cost protein food)

	Control (Rice diet)	Experimental (Rice/LCPF diet)	Significance of difference
Height ...	1.27	1.61	Sig. at 1%
Weight (pounds) ...	1.25	3.31	„ „ 0.1%
Haemoglobin (g/100cc) ...	— 0.08	0.69	„ „ 5%
Red blood cell (106/cu. mm)	— 0.08	0.22	„ „ 1%

A metabolism experiment was carried out during the middle of the feeding period to study the retention of nitrogen, calcium and phosphorus in the children. The results (Table IX and X) show that the children receiving the supplements (Indian Multipurpose Food and low-cost protein food) retained significantly larger amounts of nitrogen, calcium and phosphorus than the control children not receiving the supplement.



TABLE VIII  
*Nutritional status\* of control and experimental children*

			Rice (control)	Rice + MPF (experimental)	Rice (control)	Rice + LCPF (experimental)
Number of children	...	...	23	23	22†	20
Number of children:						
Improved	...	...	...	18	...	14
Stationary	...	...	10	5	16	5
Deteriorated	...	...	13	...	3	...

\* One boy fell sick and was found not fit for examination at the end of the experiment.  
† According to the rapid score card recommended by the Indian Council of Medical Research: Nutrition Advisory Committee 1948.

TABLE IX  
*Mean daily intake and balance of nitrogen, calcium and phosphorus of children on the rice diet and on the rice-multipurpose food diet*

					Rice (Control)	Rice + MPF (Experimental)
Nitrogen (g)	{	Intake	...	...	2.9	6.09
	{	Balance	...	...	0.45	0.96
Calcium (mg)	{	Intake	...	...	2.87	566
	{	Balance	...	...	53	195
Phosphorus (mg)	{	Intake	...	...	471	797
	{	Balance	...	...	76	164

TABLE X  
*Mean daily intake and balance of nitrogen, calcium and phosphorus of children on the rice diet and rice low-cost protein food diet*

					Rice diet (Control)	Rice — LCPF (Experimental)
Nitrogen (g)	{	Intake	...	...	6.73	9.94
	{	Balance	...	...	1.37	2.12
Calcium (mg)	{	Intake	...	...	613	853
	{	Balance	...	...	255	355
Phosphorus (mg)	{	Intake	...	...	1,144	1,483
	{	Balance	...	...	434	538



### Supplementary value of Multipurpose Food (formula C) and balanced malt food to the diets of undernourished weaned infants

A feeding trial was carried out to study the effect of a supplement of Multipurpose food (formula C) with 20 per cent added skim milk powder or balanced malt food (consisting of a blend of Jowar malt, low fat groundnut flour, puffed Bengal gram flour and skim milk powder fortified with calcium and vitamins B<sub>1</sub>, B<sub>2</sub>, A and D) on the growth and nutritional status of undernourished weaned infants.

The subjects of the experiment were fifty four weaned infants ranging in age from 9 to 20 months. The height, weight, haemoglobin and nutritional status of the children were determined according to the methods of Reddy *et al*<sup>5</sup>. On the basis of the initial height, weight and nutritional status, the weaned infants were allotted to three groups so that each group consisted of ten girls and eight boys.

The pattern of the diets consumed by the different children was more or less similar. The main cereal in the diet was milled rice. The diets included small amounts of cow's milk, vegetables, dhal and bread. In addition to their usual diet, each child in the experimental groups I and II received daily a supplement of two ounces of malt food or protein food [Multipurpose food (formula C)] and one ounce of sugar respectively. The nutritive value of the different supplements is given in Table II. In order to equalise the calorie intake in the two groups each child in the control group received two ounces of coarsely powdered rice and one ounce of sugar. The experiment lasted for a period of nine months at the end of which, height, weight, nutritional status and haemoglobin content of blood of all the children were again determined.

It will be seen from the results (Table XI) that the increase in height, weight and haemoglobin content in the case of children receiving the supplements of Multipurpose

TABLE XI  
*Increase in height, weight and haemoglobin of control and experimental children*  
(10 girls and 8 boys in each group—Experimental period—9 months)

	Control (Rice) A	Exptl (Malt food) B	Exptl (MPF) (c) C	Difference in the increase (Experimental—Control)			
				(with standard error)			
				B — A	C — A	C — B	
<b>Girls</b>							
Height (inches) ...	1.38	2.43	2.43	1.05 ± 0.25†	1.05 ± 0.25†	0.00 ± 0.25 N.S.	
Weight (pounds) ...	2.26	5.04	5.18	2.78 ± 0.30†	2.92 ± 0.30†	0.14 ± 0.30 N.S.	
Haemoglobin (g/100cc) ...	0.23	1.22	1.54	0.99 ± 0.32†	1.31 ± 0.32†	0.32 ± 0.32 N.S.	
<b>Boys</b>							
Height (inches) ...	1.69	2.15	2.51	0.46 ± 0.14	0.82 ± 0.14†	0.36 ± 0.14*	
Weight (pounds) ...	1.63	4.21	4.29	2.58 ± 0.27†	2.66 ± 0.27†	0.08 ± 0.27 N.S.	
Haemoglobin (g/100cc) ...	0.36	1.27	1.21	0.91 ± 0.18†	0.85 ± 0.18†	-0.06 ± 0.18 N.S.	

† Very highly sig. (P < 0.001).

\* Significant (P < 0.05).

† Highly sig. (P < 0.01).

N.S. = Not significant.



food (formula C) and Malt food are significantly greater than those observed in children in the control group given a supplement of rice.

## Conclusion

It is evident from the results obtained in this investigation that low-cost protein foods fortified with vitamins and minerals made from blends of edible quality groundnut flour, coconut meal and Bengal gram flours have a fairly high nutritive value and can be used as supplements to the poor dietary of a large section of the people in the country. Since protective and protein rich foods such as milk, eggs, meat and fish are in short supply in the country, the large scale manufacture and distribution of low-cost supplementary foods like the above, will help effectively in making up the deficiencies in the diets and improving the health and nutritional status of children and other vulnerable section of population in India and other technically underdeveloped countries.

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## Discussion

- Q. On how many school children have feeding trials been carried out with Indian MPF?
- A. In the first instance, under the auspices of the Madras Corporation over 200 school children were given  $\frac{1}{2}$  oz MPF as a supplement to their daily diet. Even this small supplement resulted in perceptible improvements in weight and height as compared with the controls.
- Q. How far does MPF serve to meet the nutritional requirements?
- A. About 2 oz would provide one third the minimum daily requirements of protein, vitamins, and minerals.
- Q. Is MPF being manufactured in India?
- A. At present there is no commercial production. About  $\frac{1}{2}$  ton per day is being made in the unit at the Central Food Technological Research Institute in Mysore, for experimental trials. It is proposed to set up very soon a commercial plant at Coimbatore with a production capacity of 2-3 ton per day. For this the edible quality expeller groundnut cake produced by the plant to be set up with UNICEF aid at Madras will be made use of. The MPF produced at Coimbatore will be used for feeding half a million school children as part of the school feeding programmes of the Government of Madras.



- Q. What is the effect of the roasting on the proteins in the ingredients used for making MPF ?
- A. The degree of roasting is such that the proteins are only denatured without impairing their nutritive value. This has been shown by feeding tests on albino rats.
- Q. Has MPF been used in the treatment of Kwashiorkor ?
- A. At the Nutrition Research Laboratories in Hyderabad and elsewhere under the auspices of the Indian Council of Medical Research, clinical trials have been carried out with combinations of Bengal gram and groundnut cake proteins, as such, and with varying proportions of skim milk powder. The vegetable protein blends, especially when supplemented with 20-30 per cent skim milk powder have been found to be quite effective in cases of Kwashiorkor. Similar results have been obtained in trials carried out on a smaller group of cases at the Central Food Technological Research Institute in Mysore.



# DEVELOPMENT OF PRECOOKED BALANCED PROTEIN FOOD SUITABLE FOR WEANED INFANTS

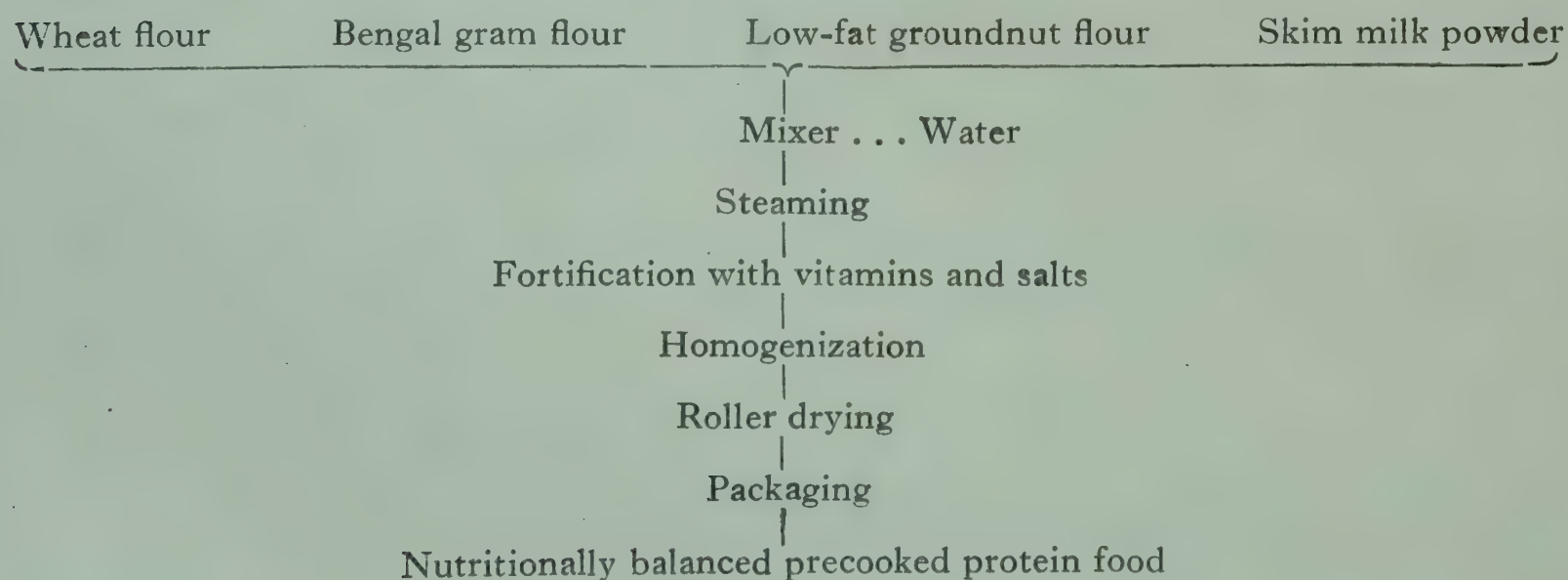
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Dietary and nutrition surveys conducted in different parts of India have shown that the diets consumed by weaned infants and young children belonging to low income groups of the population are grossly deficient in proteins, certain vitamins and minerals<sup>1</sup>. Due to the scarcity and high cost of milk and other protective foods, the children of the poorer classes after weaning are generally fed on cooked cereals or cereal gruels which have low protein contents and are deficient in certain essential vitamins and minerals. The incidence of protein malnutrition (kwashiorkor) and other deficiency diseases is quite high among the children of the poor classes<sup>2</sup>.

With a view to overcoming the shortage in the supply of milk and other protective foods in many technically under developed countries, considerable attention has been paid to the possibility of using protein rich foods of vegetable origin such as oilseeds and oilseed meals and pulses in the feeding of weaned infants and young children<sup>3</sup>. Investigations carried out by Chick and Slack<sup>4</sup> and later by Dean<sup>5</sup> have shown that a highly nutritious food can be prepared by blending barley malt extract with soya bean flour. Dean<sup>5</sup> reported that about half the milk in the diet of infants up to one year of age could be replaced by a barley malt—soya mixture and even to a greater extent in the diets of older children without affecting their growth and general health. Later investigations have shown the possibility of preparing several compositions based on cereals, oilseed meals and pulses, suitable for feeding weaned infants<sup>6, 7</sup>. Subrahmanyam *et al*<sup>6</sup> have shown that two ounces of a balanced malt food (a blend of sorghum malt, low-fat groundnut flour, Bengal gram flour and skim milk powder) or Indian MPF (a blend of groundnut flour 60 parts, Bengal gram flour 20 parts and skim milk powder 20 parts fortified with vitamins and minerals) in the diet form effective supplements to the diets of weaned infants aged 9-20 months. Scrimshaw *et al*<sup>7</sup>.

FIG. 1

*Flow sheet of the process for the manufacture of precooked weaning food*



developed a vegetable mixture known as INCAP mixture 9B (blend of corn, 29 parts, sorghum, 29 parts, cottonseed meal, 38 parts, dry Torula yeast, 3 parts, calcium carbonate, 1 part and vitamin A, 10,000 I.U.) which was found to be very effective in the treatment of kwashiorkor in children. The present paper gives details of the process and project costs for the preparation of a low cost precooked protein food suitable for feeding weaned infants.



## Process

The flow sheet of the process for the manufacture of precooked weaning food is given in Fig. 1. The process consists of the following steps.

### Blending of cereal, pulse and oil seed flours

Refined wheat flour, puffed Bengal gram flour and low-fat groundnut flour were mixed thoroughly in a mechanical mixer. Puffed Bengal gram flour was prepared by powdering, cleaned puffed Bengal gram in a flour mill. Low-fat groundnut flour used in the preparation of the protein food was prepared according to the procedure of Subrahmanyam *et al*<sup>8</sup>.

### Cooking of the blend

The blend of refined wheat flour, puffed Bengal gram flour and low-fat groundnut flour was mixed with eight times its amount of water and then cooked in steam for half an hour.

### Fortification

The cooked blend was fortified with calcium salts (calcium carbonate or phosphate) and vitamins A and D, thiamine and riboflavin. The different vitamins and minerals were made into a premix and then added. The mixture was thoroughly mixed in a triple homogenizer at 1,000 lb/sq. in. pressure.

### Drying and packing

The material was fed on to an atmospheric stainless steel twin roller drier working at 40-45 lb steam pressure and 2-3 revolutions per minute. The material dried in the form of a continuous band. The dried material was powdered to a coarse powder and packed in 1 lb polythene bags. These bags were repacked in 4 gallon tin containers with lever lids. The chemical composition of the precooked protein food as compared to that of skim milk and whole milk powders is given in Table I.

TABLE I  
Composition of precooked protein food  
(Values for 100 g)

			Precooked protein food	Whole milk powder	Skim milk powder
Protein (N $\times$ 6.25) (g)	...	...	32.2	25.8	38.0
Fat (g)	...	...	5.6	26.7	0.1
Carbohydrate (g)	...	...	46.4	38.0	51.0
Calcium (g)	...	...	0.86	0.95	1.37
Phosphorus (g)	...	...	0.53	0.73	1.00
Iron (mg)	...	...	7.2	0.6	1.4
Thiamine (mg)	...	...	2.0	0.31	0.45
Riboflavin (mg)	...	...	3.2	1.36	1.64
Nicotinic acid (mg)	...	...	9.5	0.8	1.0
Vitamin A (I.U.)	...	...	3,000	1,400	nil
„ D (I.U.)	...	...	500	...	nil







## Nutritive value

The protein efficiency ratio of the proteins of the precooked protein food was estimated by the rat growth method of Osborne, Mendel and Ferry. The results are given in Table III.

TABLE III  
*Protein efficiency ratio of the proteins of the precooked balanced protein food*  
(at 10% level)

Diet	Protein efficiency ratio	
	4 weeks	8 weeks
Precooked balanced protein food*	1.86	1.56

\* Refined wheat flour (maida) 25 parts.  
Groundnut flour 45 parts.  
Bengal gram flour 30 parts.

The results show that the proteins of the food are of fairly high nutritive value. Since the food is also rich in other nutrients like minerals and vitamins which are also deficient in the diets of weaned children, a daily intake of one ounce of the food will form a good supplement, to the diets of weaned infants, providing one third the daily requirements of all nutrients. The cost of the product is also low and within the means of the low income groups. Further investigations on the nutritive value of the product and feeding trials with weaned infants will be carried out shortly.

## Shelf life

When packed in hermetically sealed cans, the shelf life of the precooked protein food was satisfactory for a period of 9 months at 37°C and more than one year at room temperature (25-30°C).

## Consumer acceptability

The product when mixed with six times the amount of boiling water and sweetened with sugar yielded a tasty porridge. Organoleptic trials carried out on 15 weaned infants aged 12-18 months for a period of one week showed that the product was quite acceptable.

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# THE NUTRITIONAL VALUE OF FISH FLOUR

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The dramatic increase in world population and widespread prevalence of protein malnutrition in many areas of the world, have prompted investigations on the nutritional value of food obtained from marine sources. It appears certain that greater use of the food resources of the seas and inland water of the world will be made in the future.

Possibilities for increasing the supply and consumption of fish depend not only upon scientific management of fish resources<sup>1</sup> but also on technological advances in preservation and distribution. Frozen or canned fish are, in general, too expensive for use in technically underdeveloped countries, where protein malnutrition is most prevalent and population pressures are most severe. Other more traditional methods of preservation, such as smoking, drying or salting, are widely used,<sup>2</sup> but fish so processed do not have a long storage life, especially under tropical conditions. Recently, processes have been developed by which fresh fish, or high quality fish meal can be treated to remove the moisture and lipid, yielding a stable high protein 'flour', which if desired, can be made practically odourless. The purpose of this report is to review briefly present knowledge of the nutritional value of fish flour.

Several processes have been used to produce fish flour. In the one described by Guttman and Vandenheuvel<sup>3</sup>, washed fresh offal, consisting of the trimmings from filleting tables, excluding the heads, from fish, such as cod, haddock and hake was heated with dilute polyphosphoric acid to 75-78°C for 30 minutes. The slurry was then filtered and the resulting press-cake washed with hot water. Lipid and water were extracted from the press-cake with hot isopropanol. The press-cake was then screened to remove the skin and bones, dried, ground and residual solvent was removed under reduced pressure. The yield was 10 parts of finished flour from 100 parts of offal. The Dabsch process for producing fish flour, used in the pilot fish flour plant supported by UNICEF in Chile, involves azeotropic extraction with petroleum ether, followed by deodorization by means of repeated washings with ethanol<sup>4</sup>. Another process, the Vogel process, is notable primarily as deodorizing and deflavouring procedure. Fat-free material is treated with acid or alkali, plus organic solvents, and the final product is dried at reduced temperature<sup>4</sup>. In the so-called Viobin process<sup>5</sup>, dehydration and lipid extraction are carried out simultaneously, using ethylene dichloride. Other processes for preparing edible fish flour have also been developed, among which should be noted the South African process, involving ethanol extraction, followed by drying with hot air, with or without steam stripping.

The contribution made by fish to world food supplies is predominantly that of protein. Since the nutritional value of fish flour is limited by that of the fish from which the flour is produced, it would appear appropriate, in a review such as this, to discuss briefly the nutritional value of fish protein. In comparison with other proteins of animal origin,



relatively little work has been done on the nutritive value of fish proteins. Deuel *et al*<sup>6</sup>. prepared fish protein from edible fish muscle by isoelectric precipitation, followed by purification with organic solvents to produce a light-coloured, practically odourless powder. Mackerel, sardine and tuna muscle proteins were found to be superior to casein in promoting growth in weanling rats. Mackerel protein was superior to casein, as measured by stimulation of haemoglobin production in anaemic rats and was equally effective as casein in stimulating plasma protein synthesis.

The findings of Beveridge<sup>7</sup> further demonstrate that fish protein is of good nutritional value. Beveridge determined the biological value of the crude flesh proteins of herring, lingcod, halibut, lemon sole and white spring salmon, by means of protein efficiency ratio and nitrogen retention measurements in growing rats. The fish were prepared in the form of fillets or steaks, cooked by steaming for 1 hour at 99-100°C and dried, together with the cooking liquors, at a low temperature. For comparison, beef flesh prepared in the same manner as the fish, casein, and dried egg albumin were also tested. The values found for the fish products, along with those for beef, are summarized in Table I. The

TABLE I  
*Biological value of fish protein\**

Source of protein	Protein efficiency ratio g gain/g protein	Nitrogen retention %
Lingcod	3.11	56.9
Halibut	3.15	57.7
Lemon sole	3.04	55.4
Salmon	3.12	55.0
Beef	2.80	50.6

\* Taken from data of Beveridge<sup>7</sup>.

figures obtained for the flesh proteins from the four types of fish were similar, and significantly higher than those found for beef. Millares and Fellers<sup>8</sup> reported that the proteins of beef, chicken and fish were of similar nutritional value for growing rats. Lopez-Matas and Fellers<sup>9</sup> concluded that the nutritional values of swordfish muscle, beef and chicken were approximately equal. The essential amino acid content of swordfish muscle was found to be similar to that of beef. Miller<sup>10</sup> studied the nutritional value of extracted cod proteins prepared in the laboratory. Net protein utilization values of approximately 80 per cent were obtained, higher than those found for casein or meat proteins. An extensive review of the nutritional value and amino acid composition of fish and fish products has recently been published<sup>11</sup> and indicates that, in general, fish proteins contain adequate amounts of the essential amino acids, in this respect resembling most other proteins from animal muscle.

Although the nutritive value of fish flour has been examined by several investigators, it is difficult, in many instances, to determine if the product tested was an edible fish flour or a fish meal made from fish by-products and designed for animal feeding. Miller<sup>10</sup> found the percentage net protein utilization of a deodorized South African fish flour to be 67, significantly less than that found for cod protein preparations produced in the laboratory. As reported by FAO<sup>4</sup>, Bender has studied the net protein utilization of a considerable number of fish flour samples prepared from a variety of species of fish by various pro-



TABLE II  
*Biological value of various fish flour samples\**

Raw material	Net protein utilization†	Digestibility %	Biological value %
Lean fish	49	93	53
Cod	65	95	69
Cod fillets	64	95	67
Herring	74	93	79
Lean fish	73	93	78
Lean fish	77	94	82
Lean fish	77	96	80
Fatty fish	29	81	36
Fatty fish	31	68	46
Fatty fish	42	71	59
Semi-lean fish	67	94	71
Semi-lean fish	55	96	58
Herring	56	94	60
Sardines	70	95	74
Gutted haddock	69	95	73
Whole cod	67	95	71
Cod	64	95	67

\* Taken from data of FAO<sup>4</sup>. † Digestibility × Biological Value.

TABLE III  
*Nutritional value of fish flour fed at three different protein levels\**

	Casein			Fish flour		
	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Diet protein, %	7	10	15	7	10	15
Weight gain, g	53±10†	99±17	152±21	106±9	148±10	162±13
Protein efficiency ratio, g gain/g protein	2.86±0.81	3.04±0.30	2.74±0.21	4.19±0.35	3.80±0.19	2.71±0.16
Liver lipids, %	5.02±0.62	4.50±1.07	5.67±1.18	5.50±1.15	5.14±0.64	5.47±0.80

\* Taken from data of Morrison and Campbell<sup>12</sup>. † Standard deviation.

cesses. The values obtained are summarized in Table II. Digestibility coefficients varied from 68 to 96 per cent and net protein utilization values varied from 29 to 77 per cent. It is not possible from the data presented, to separate the effects of processing conditions from those of species of fish.

We have recently had occasion<sup>12</sup> to examine the nutritional value of a sample of Canadian fish flour produced from cod by the procedure of Guttman and Vandenheuvel, described above. The product used was a fat-free greyish powder, containing 90 per cent protein (N×6.25). Male weanling rats received otherwise adequate diets containing 7, 10 or 15 per cent protein supplied by casein or fish flour. Protein efficiency ratio (PER) values were calculated after 4 weeks by the method of Chapman *et al.*,<sup>13</sup> and the results are summarized in Table III. PER values found for 7 per cent or 10 per cent fish protein were significantly higher than those for comparable levels of casein, but similar values were found for both proteins at the 15 per cent level. The kidneys of the animals given diets containing



TABLE IV  
*Effect of fish flour on nutritional value of white bread\**

	Diet 1 Casein	Diet 2 Bread	Diet 3 Bread + 10% fish flour	Diet 4 Bread + 4.2% milk solids	Diet 5 Bread + 4.2% milk solids + 10% fish flour
Weight gain, g ...	109 ± 14†	23 ± 8	145 ± 16	43 ± 10	130 ± 13
Protein efficiency ratio, g gain/g protein ...	3.49 ± 0.14	1.30 ± 0.21	3.87 ± 0.13	1.97 ± 0.27	3.59 ± 0.18
Liver lipids, % ...	5.48 ± 0.30	8.38 ± 0.87	6.17 ± 1.04	9.20 ± 2.10	6.30 ± 1.15
Liver cholesterol, mg/g wet tissue ...	2.50 ± 1.38	3.71 ± 0.57	2.86 ± 0.89	2.94 ± 1.18	2.13 ± 1.31
Lysine, g/16 g N ...	...	2.43	6.32	3.16	5.86

\* Taken from data of Morrison and Campbell<sup>12</sup>.

† Standard deviation.

15 per cent protein were significantly heavier than those of the animals fed the other diets, but the level or source of dietary protein had no significant effects on liver or adrenal weights or liver lipid levels.

The high lysine content of fish flour<sup>3,14</sup> indicated that it should be of particular value in supplementing cereal diets deficient in this essential amino acid. Sure<sup>15,16</sup> reported that addition of small amounts of fish flour to the diets of growing rats increased the PER values of a number of cereal grains including rye, wheat, corn and millet. In additional studies, we determined the effects of fish flour on the nutritional value of white bread. Four samples of enriched white bread were made, two of which contained 4.2 per cent milk solids, whereas the other two samples were made with water. Prior to baking, 10 per cent fish flour was added to one of the samples made with milk and to one of those made with water, in place of white flour. After baking, the bread samples were sliced, dried at room temperature, ground and fed to groups of male weanling rats at the 10 per cent protein level, in an otherwise adequate diet<sup>13</sup>. The results obtained are shown in Table IV. The bread samples which contained 10 per cent fish flour showed reduced loaf volume, were slightly darker in colour, and had a somewhat 'rubbery' texture, as compared to the normal breads, but the smell and taste were not noticeably affected. Addition of fish flour to bread made with or without milk increased the PER values by 82 per cent and 198 per cent respectively. Liver lipid levels were significantly lower in the animals which received diets without fish flour. Fish flour tended to reduce liver cholesterol levels, although the results were not statistically significant. Addition of fish flour to the bread samples made with or without milk markedly increased their lysine contents. It was found that the effects of fish flour on PER of bread diets could be explained on the basis of its contribution of lysine to the diet.

In further studies, the comparative values of fish flour and non-fat milk solids as supplements to white bread diets were determined. Bread samples were prepared which contained 3, 6 or 9 per cent non-fat milk solids, or equivalent amounts of protein from fish



flour. The samples were dried, ground and fed to groups of male weanling rats at the 10 per cent protein level in an otherwise adequate diet. PER values found after 4 weeks are summarized in Table V. At all three levels tested, fish flour increased the PER values of bread to a significantly greater extent than did an equivalent amount of protein from milk.

In other experiments, now in progress, it has been found that some samples of fish flour do not have as high nutritive value as the sample of fish flour tested by Morrison and Campbell<sup>12</sup>. Unavailability of lysine, and perhaps other amino acids as well, appears to be a problem in certain fish flours and is probably related to processing damage. It is well known<sup>17, 18</sup> that processing conditions influence the nutritional value of fish meals for animal feeding.

TABLE V  
*Comparative value of protein from fish flour and  
non-fat milk solids in supplementing white bread*

Diet	Protein efficiency ratio (g gain/g protein)
Casein	2.77
Bread	0.66
Bread + 3% non-fat milk solids	0.99
Bread + 1.15% fish flour	1.49
Bread + 6% non-fat milk solids	0.97
Bread + 2.30% fish flour	1.53
Bread + 9% non-fat milk solids	1.45
Bread + 3.45% fish flour	1.70

Fish flour has also been tested in human diets, although many of the studies conducted were in reality acceptability trials. In parts of Africa and South East Asia, fish flour with residual fish flavour is preferred, whereas in other countries, such as Chile, a bland, flavourless flour is desired. Less expensive processes can be used for production of flavoured fish flours, than for the production of deodorized products. Autret and van Veen<sup>19</sup> reported the results of experiments conducted in several countries under the auspices of FAO. In trials conducted in Chile, fish flour (sample SA 2 of Miller<sup>10</sup>) was added to a variety of foods, including soup, beans, beet leaves pie, potatoes, crackers, cake and bread. Although some foods with fish flour added were not acceptable, beet leaves pie, crackers, coffee cake and bread supplemented with fish flour were well accepted. In a subsequent more extensive study, involving 140 Chilean school children, bread was made containing 10 per cent fish flour. Although the fish flour supplemented bread was somewhat darker in colour than normal bread, it was well accepted.

The nutritional value of fish flour in human diets has also been studied in other countries, including Belgian Congo, India, Pakistan, Peru, Senegal, Columbia, Brazil, Cameroons, Ecuador, French Equatorial Africa, French Sudan, French West Africa, Mexico and Turkey<sup>4</sup>. The studies in French West Africa are worthy of special mention, because they involved trials with children suffering from kwashiorkor<sup>20</sup>. Fish flour was combined with millet or peanuts to give a food which, at the levels used, increased the protein content of the diet by 70-80 per cent. A mixture of millet, peanut press cake and fish flour appeared to increase weight gain in children suffering from kwashiorkor, as compared to those given a supplement of millet without peanuts and fish.



In summary, the problems of protein malnutrition and population pressure have focused increasing attention on the importance of fish in world food supplies. Fish flour, containing about 90 per cent protein, is probably the most convenient and acceptable form for utilizing fish protein, particularly in tropical areas. When properly prepared, fish flour is stable, practically odourless and tasteless and has been found acceptable in a variety of food uses. The protein is of high quality and, because of its relatively high lysine content, is of particular value in supplementing cereal diets. It may be concluded that fish flour can provide a valuable contribution to the food supplies of any country where the protein intake is limited in quality or quantity.

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## SOME PROTEIN-RICH PROCESSED FOODS FROM FISH

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Fish is a good protein-rich food. In general, fish proteins contain all the essential amino acids in adequate amounts and in balanced proportions<sup>1</sup> and in this respect, resemble other proteins of animal origin<sup>2</sup>. As a class, fish proteins are a valuable source of lysine and methionine.<sup>3-6</sup> Fish proteins possess high digestibility<sup>7</sup>, biological value<sup>8</sup>, and growth-promoting capacity<sup>9</sup>.

Since fish is of highly perishable nature, larger catches of fish during the glut season have to be processed to enable efficient distribution to interior areas far from the fishing centres. Large quantities of fish meal are produced from surplus fish, inedible fish or filleting waste. However, fish meal cannot be stored for long periods; it must be used soon after it is made. It soon goes rancid<sup>10, 11</sup>, and rancid fish poses a distinct problem of toxicity.

Fish flour is the popular name for fish meal prepared with sufficient care to render it fit for human consumption. Edible fish flour contains 75-80 per cent protein of high biological value. Its cost of production is reported to compare favourably with that of other protein-rich foods of animal origin. Fish flour, being a highly concentrated source of protein, offers special advantage for fortifying foods with protein of high biological value. Keen interest has been evinced in many countries<sup>12</sup> in edible fish flour as a source to enrich protein-deficient diets.

### Fish flour

In the VioBin Corporation Process<sup>13</sup> for the preparation of deodorized fish flour, fish flesh is simultaneously subjected to dehydration and defatting with a suitable solvent having a low azeotropic distillation temperature. Proteins can thus be recovered undenatured and other valuable constituents are similarly retained. Based on a similar principle of azeotropic distillation, the Dabsch Process advocates the use of benzene of boiling point 105-110°C, for fresh fish. For defatting commercial fish meal, benzene of lower boiling point is used followed by deodorization with 96 per cent ethyl alcohol. A Japanese process<sup>14</sup> describes the use of 94 per cent ethyl alcohol for removing disagreeable flavour from dried fish. In a French process<sup>15</sup> fresh fish pulp extracted exhaustively in the cold or at an elevated temperature with acetone gives practically anhydrous residue free from any odour. A South African process<sup>16</sup> describes the use of 90 per cent industrial alcohol of 96 per cent strength and 10 per cent ethyl acetate for extracting fish meal. The ratio of solvent to meal is about 2.6:1 on weight basis.

The Vogel process<sup>17</sup> describes extraction of ground fresh fish or fish meal with alcohol to low fat content. The product is then treated at 20-80°C with alkali which destroys the odoriferous compounds without deleterious effect on the essential amino acids. The mixture is adjusted to the isoelectric point (pH 5.4), the solids removed, washed with ethyl alcohol, and dried at 60°C. The product obtained is almost white, tasteless and odourless.

In a Russian process<sup>18</sup> the finely ground raw material is digested with 0.25 to 0.5 per cent NaOH at room temperature for 15-17 hours. The protein from the alkali extract



is precipitated by the addition of acetic acid, washed, pressed, defatted and deodorized by extraction with alcohol in the case of lean fish and ethylene dichloride in the case of medium and high-fat fishes.

A Canadian process<sup>19</sup> describes preparation of edible fish flour from cod and haddock fillet waste. The material is heated with dilute acid, washed and pressed. The resultant press cake is extracted with isopropyl alcohol when an almost white odourless, tasteless flour-like product is obtained.

Edible fish proteins, free from fishy smell, have been prepared from skate, shark, ray, etc., by a process similar to that described for the manufacture of Wicking-Eiweiss—a German product<sup>20</sup>.

A method has been developed by Pillai<sup>21, 22</sup> for preparing fish flour by fermenting fish flesh in the presence of defatted butter milk and subsequently treating it in the same way as in the reduction process. The method is found to be particularly suited to elasmobranch fishes where the body oil content is very low. It is reported that the product so obtained does not impart fish flavour to food preparations when quantities up to 15 per cent by weight of the flour are used.

A detailed method describing the use of acetic acid and alcohol for the isolation and purification of fish proteins on a laboratory scale has been worked out by Valanju and Sohoni<sup>23</sup>. The proteins prepared are light-yellow to pale-brown in colour and are almost without odour. The method is similar to that described in 'Certain Aspects of German Fishing'<sup>24</sup>.

A method of preparation of edible proteins from shark and skates<sup>25</sup> consists in the extraction of the proteins with dilute alkali and precipitation of the protein from the extract with acetic acid.

A major portion of the total catch of oil-sardine (*Clupea longiceps*) is used for the extraction of oil and the press cake as fertilizer in India. Our studies<sup>26</sup> indicate the feasibility of processing oil sardine, in an efficient manner, to obtain good quality oil and deodorized fish flour for human consumption. Oil sardines are dressed to separate entrails consisting of head, tail and viscera from the body meat. These two portions are separately cooked in open pans on fire till the meat becomes soft and then pressed in gunny bags using hand-driven screw press. For solvent extraction, the cake is dried to a moisture content of about 5 per cent at a circulating temperature of 60°C or in the sun. The dry material is mechanically broken up into small pieces and then extracted with ethyl alcohol. The last traces of solvent are removed in a vacuum shelf drier and the material then finely ground in a micro-pulverizer. From every 100 lb of the fresh sardine, approximately, 10 lb of deodorized fish flour for human consumption and 8 lb of poultry feed can be recovered. (Table I and II).

TABLE I  
Yield of various products on processing 100 lb oil sardine

Oil sardine (starting material)		100 lb
Entrails (viscera, head etc.,) (fat, 28.8%)	} After dressing	{ 45 lb
Muscle portion (fat, 12.1%)		
Pressed muscle cake (moisture, 50.0%)		21.8 lb
Pressed entrail cake (moisture, 50.0%)		16.5 lb
Alcohol extracted fish flour (protein, 81.5%)		10.0 lb
Entrail cake (protein, 41.0%)		8.0 lb



TABLE II  
*Approximate chemical composition*

Description of the material	Percentage composition			
	Moisture	Fat	Protein	Ash
Pressed body cake ...	49.7	6.2	40.8	5.2
Pressed entrail cake ...	50.0	7.75	21.5	18.0
Alcohol extracted fish flour (from body cake) ...	3.5	0.114	81.5	10.1
Entrail cake ...	5.0	15.5	41.0	35.9

TABLE III  
*Effect of different treatments on removal of urea from shark flesh*

Treatment	Urea content expressed as mgN%
1. Shark flesh dried at 45-50°C. ... ..	1,107.8
2. Shark flesh steamed for 15 minutes at 2-lb pressure ...	1,090.2
3. Steamed shark flesh blended with soyabean flour in the ratio of 8:1 and then dried in sun ... ..	460.0
4. Steamed shark repeatedly washed with water ...	21.0
5. Steamed shark repeatedly washed with dilute HCl at pH 5.0 ... ..	30.8

Fish like skate, shark, ribbon, etc., contain relatively large amounts of non-protein nitrogenous compounds including urea and these are responsible for the undesirable taste in the processed material. It has been shown that urea is acceptable in feeds for ruminants and even for them large amounts are considered toxic<sup>27</sup>. Investigations<sup>28</sup> are in progress here to evolve suitable methods for removal of urea from shark muscle. It was observed that steaming of shark muscle just to coagulate it followed by repeated washing with water gave best results as regards removal of urea. The time of dehydration could be minimised to half by steaming the tissue prior to drying. The fine powder was white in colour practically free from off-flavour. Another method that could be employed to remove urea by about 50 per cent is to blend steamed shark muscle with soyabean powder in the ratio of 8:1 respectively. In the finished product any fishy odour, is masked by the beany flavour. (Table III).

**Fish enriched products**

Efforts have so far been directed towards preparing fat-free and completely deodorized fish flour. If, however, the fat could be retained without undergoing any change, it would cut down the cost of producing fish-enriched products. Our investigations have shown that enriched fish flour obtained by blending lean fish and starchy material containing



antioxidant combination, can be stored for long periods. It will serve as vehicle for preparation of various food products. The method is a modification of the preparation of fish enriched maize flour by the Atlas-De Vries process<sup>28</sup>. A good quality fish macaroni<sup>29</sup> can be prepared by blending equal quantities of fish flesh containing antioxidant combination (Tenox II, i.e. 0.01 per cent B.H.A. + 0.003 per cent propyl gallate + 0.006 per cent citric acid on dry basis) and starchy material like wheat flour (semolina) or by partial gelatinization of a part of tapioca with hot water followed by incorporation of wheat semolina. The blended materials are passed through a press to obtain the product in the form of tubes. The extruded product is dried in a cabinet drier at 60°C, for about 3-4 hours till the moisture content is reduced to about 5 per cent. The finished product is cheap and highly nutritious containing about 15-20 per cent protein of high biological value. It can be adopted on a cottage scale with minimum of equipment. Seasoned fish macaroni containing salt, chillies and tamarind extract is not only tasty but also has a better keeping quality because of the known preservative effect of added spices. The product may also be enriched with minerals and vitamins. (Table IV).

TABLE IV  
*Cooking quality of fish macaroni\**

Composition							Fish flesh 40% Semolina 60%
Volume of 100 g of macaroni products							82.0 Cm. <sup>3</sup>
Cooking time	...	...	...	...	...	...	18 min.
Water absorbed in g	...	...	...	...	...	...	210
Volume of cooked macaroni products (Cm. <sup>3</sup> )	...	...	...	...	...	...	256
Increase in volume	...	...	...	...	...	...	2.9
Solid substances lost to water in percentages	...	...	...	...	...	...	5.9
Cooking quality	...	...	...	...	...	...	Very good

\* Extruded through Buhlers press and dried in a humidity-controlled drier.

Based on the same principle, fish cakes<sup>30</sup>, now popular in the United States, consist of steamed fish muscle blended with peeled and seasoned mashed potatoes. In this preparation, mashed potatoes are used as starchy material instead of tapioca used for fish macaroni.

Fish proteins adequately supplement human diets<sup>31-33</sup> particularly of the vulnerable sections of the population and fish flour can be easily incorporated in wheat flour and maize meal. It is ideal for use as a protective protein-food in the diet of infants, who have been shown to tolerate it even at a very young age<sup>32</sup>. The value of fish protein in the prevention and treatment of kwashiorkor<sup>34-35</sup> has been recognized and feeding experiments with children using recipes containing fish flour have been carried out<sup>33-34</sup>. It has been used as an enriching component in bread<sup>32</sup>, biscuits<sup>34</sup>, cakes<sup>32</sup>, sweets<sup>33</sup>, soups and gruel<sup>34</sup>. Incorporation of fish flour up to 10 per cent does not appreciably alter the taste and appearance of bread<sup>32</sup>.



## Conclusion

Fish flour offers itself as a potent food against protein malnutrition. Several processes have been developed by which either fresh fish or dehydrated fish meal can be defatted, deodorized and finished as an almost tasteless, odourless and nearly white flour. Clinical and consumer acceptability trials should be carried out with these products on children for long periods.

Important considerations in any process developed for the purpose should be the complete removal of fishy odour with minimum damage to the protein during processing and non-reversion of flavour during storage.

A number of fish meals and deodorized fish flours intended for human consumption when examined<sup>36</sup> for overall nutritive value showed net protein utilization factor ranging from 18 to 80 thereby indicating the widely varying extent of damage resulting from the defatting and deodorization when fish meals are converted to edible fish flours. Therefore more exhaustive investigations into the details of promising processes are needed. The toxicity resulting from residual traces of solvents in finished products has also to be carefully examined.

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# PREPARATION OF CHEDDAR AND PROCESSED CHEESE USING THE MILK CLOTTING ENZYME FROM *FICUS CARICA* L.

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Cheese is a protein rich food and a valuable supplement to the majority of Indian diets which are deficient in protein. There is very little commercial production of this valuable protective food in the country. Imported cheese is very expensive. Steps should therefore be taken to increase the indigenous production of cheese.

The use of rennin from calf mucosa in cheese making is well known. At present there is restriction in the country, on the import of animal rennet. Also, there is objection by a section of people to the consumption of cheese made with animal rennet. These difficulties could be overcome and cheese made increasingly popular, if it can be manufactured with vegetable rennet.

Investigations<sup>1, 2</sup> carried out to replace animal rennet with milk clotting enzymes from plant sources have shown that the enzyme from the latex of *Ficus carica* L. would be suitable for cheese making. Studies on the preparation of this milk clotting enzyme, its purification and physico-chemical properties (including milk clotting) have been reported<sup>2, 3</sup>. However, its utilization for manufacture of cheese has not been investigated. In the present studies attempts have been made to manufacture cheddar and processed cheese of acceptable quality with vegetable rennet.

## Experimental

Fig latex required for the studies was procured from the Mysore Govt Fig Orchard at Srirangapattana. The milk clotting enzyme from the latex was processed in the laboratory as described by previous workers<sup>2</sup>. The milk clotting activity of the enzyme was checked by the method of Krishnamurthi and Subrahmanyam<sup>2</sup>. Maximum clotting activity was obtained by using 2 ml of a 0.1 per cent aqueous solution of the enzyme extract per 100 ml of cheese milk.

## Cheddar cheese

The important steps in the manufacture of cheese with vegetable rennet are essentially the same as the conventional cheddar cheese manufacture<sup>4</sup> except for the slightly longer renneting time. Based on the results of laboratory experiments, large scale trials were carried out at the Kaira Co-operative Milk Producers Union at Anand and at the Southern Regional Station of the National Dairy Research Institute, Bangalore. About 2,500 lb of pasteurized milk (cow and buffalo) were converted into cheese. Cheese made with animal rennet served as the control. The cheeses were ripened at 5°C and R.H. of 75-80 per cent, for about 6 months. The typical manufacturing data for the vegetable and animal rennet cheeses are given in Table I.



TABLE I

Milk taken lb	Fat in milk %	Yield of green cheese		Fat lost in whey %	Yield of ripened cheese (3 weeks)		Duration of manufacture (green cheese)		Per cent yield of ripened cheese (3 weeks)
		lb	Oz		lb	Oz	Hr	Min.	
100	4.18	12	14	<i>Cow's milk</i> <i>Vegetable rennet</i> 0.17	10	12	5	55	10.76
				<i>Animal rennet</i> 0.15					
100	4.30	13	11	<i>Buffalo milk</i> <i>Vegetable rennet</i> 0.40	11	14½	5	43	11.92
84½	4.2	10	13	<i>Animal rennet</i> 0.23	10	6	5	35	10.37
85	4.10	12	11	0.23	12	0	5	38	12.00

Acid development is very important in cheese manufacture because of the desirable changes it brings about. Figure 1 shows that in general the acid development is most rapid during the interval between dipping and milling of the curd; no significant differences in the acidity curves were observed for different types of cheese.

A comparison of the manufacturing data shows that the duration of manufacture of vegetable and animal rennet cheese is of the same order. The loss of fat in 'whey' of cow milk cheese with vegetable rennet (0.17 per cent) is slightly higher than that in animal rennet cheese whey (0.15 per cent). Also, the yield of vegetable rennet cheese from cow milk (10.75 per cent) is less than the corresponding yield with animal rennet (11.92 per cent). The losses of fat in whey of buffalo milk cheese with vegetable and animal rennets are 0.40 per cent and 0.23 per cent respectively. On account of the fairly high loss of fat during manufacture, buffalo milk cheese with vegetable rennet gave a lesser out-turn (10.37 per cent), than that with animal rennet (12.0 per cent). The higher loss of fat during cheese manufacture and consequent lower yields of cheese with vegetable rennet may probably be due to the differences in the firmness of cheese curds with animal and vegetable rennets.

Cheeses were analysed by standard methods and the compositions are given in Table II.

The compositions of these cheeses are well within the standards prescribed for cheddar cheese, there being no significant differences between the compositions of cheeses made with vegetable and animal rennets.

The organoleptic evaluation of cheese after 3 and 6 months of ripening is given in Table III.



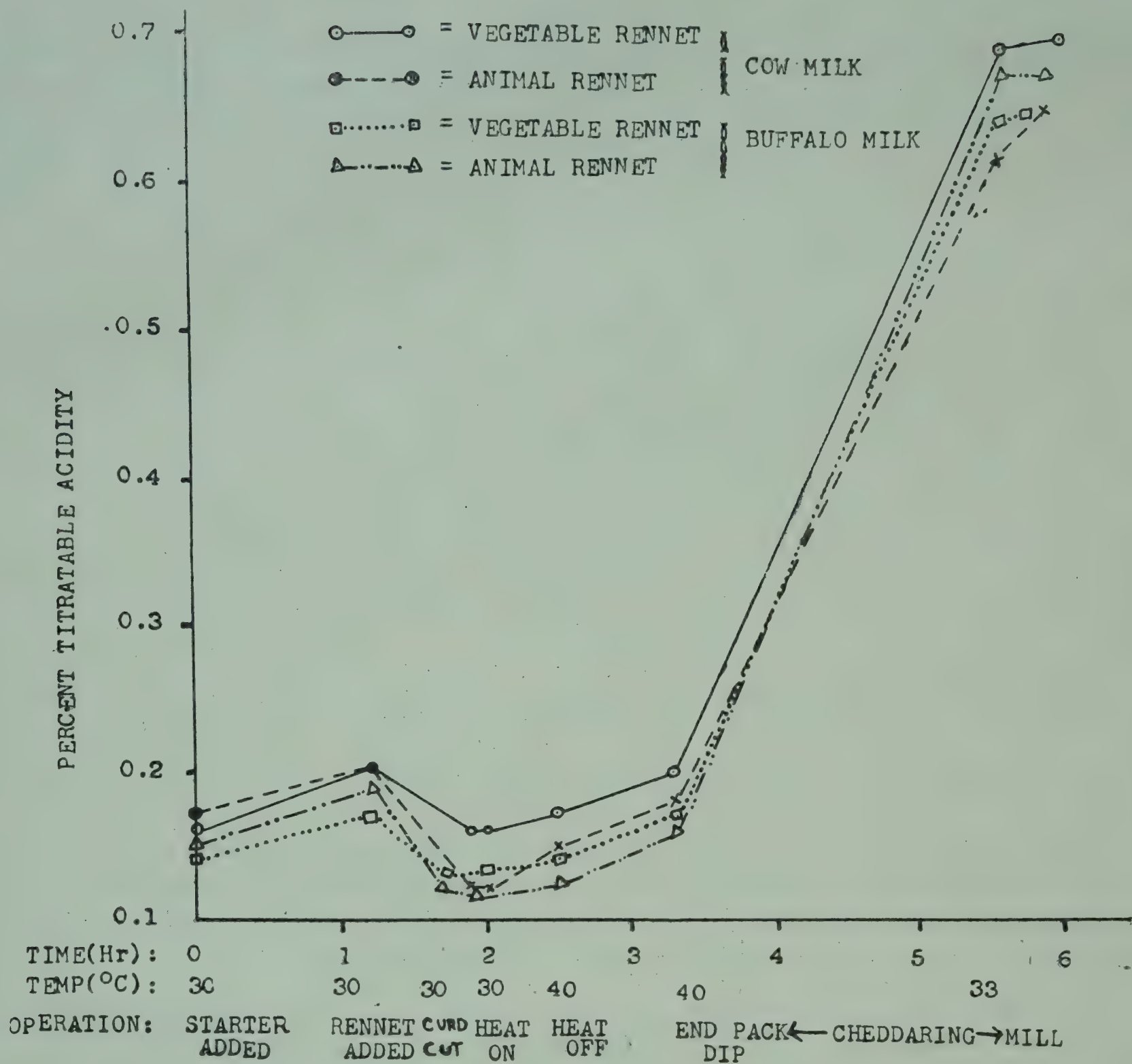


FIG. 1. ACID PRODUCTION IN CHEDDAR CHEESE

TABLE II  
*Composition of cheese*

Kind of milk	Type of rennet used	Moisture %	Total Protein (N × 6.38) %	Total fat %		pH	Ash%
				In cheese mass	In dry matter		
Cow	Vegetable	37.0	23.34	31.75	50.27	5.20	4.99
	Animal	35.2	24.47	32.58	50.33	5.28	5.06
Buffalo	Vegetable	35.8	23.88	31.82	50.10	5.32	5.86
	Animal	36.0	23.78	30.99	49.95	5.35	5.79



TABLE III  
Cheese score card

Milk				Flavour		Body and texture		Finish		Colour		Total Score		Type of rennet used
				45*		30*		15*		10*		100*		
				A	B	A	B	A	B	A	B	A	B	
Cow Milk	...	...	...	19	26	18	16	9	12	7	8	53	62	Vegetable
				25	28	19	19	8	11	9	7	61	65	Animal
Buffalo Milk	...	...	...	22	28	15	20	9	13	8	8	54	69	Vegetable
				26	31	20	18	9	12	10	7	65	68	Animal

\* Perfect score.                      A = 3 Months.                      B = 6 Months.

All cheeses showed improvement in organoleptic qualities during ripening, this being more marked with vegetable rennet cheese (cow and buffalo). Some of the batches of vegetable rennet cheese had slight bitter taste in the earlier stages of ripening which became imperceptible after prolonged ripening. This transient bitter flavour may probably be due to the larger accumulation of bitter tasting peptones<sup>5</sup> not yet broken down to amino acids. As protein breakdown products play a prominent role in the development of flavour in cheese during ripening, the NPN and proteose nitrogens were determined at the early and final stages of ripening as shown in Table IV.

TABLE IV  
Changes in protein during ripening of cheese

Type of milk				Kind of rennet	NPN %		Proteose N %	
					Early	Final	Early	Final
Cow	...	...	...	Vegetable	...	1.29	1.51	1.13
				Animal	...	0.55	0.79	0.75
Buffalo	...	...	...	Vegetable	...	1.02	1.24	1.24
				Animal	...	0.51	0.72	0.80

The vegetable rennet cheese showed an increase in NPN and a decrease in proteose nitrogen as ripening advanced. This kind of protein breakdown may also be an indication that larger quantities of bitter tasting principles formed in the earlier stages of ripening are subsequently broken down to further simpler products that may not impart bitterness to the product.



## Processed cheese

Preliminary experiments have been carried out to produce processed cheese of acceptable quality from cheese made with vegetable rennet. About 40 lb of vegetable rennet cheese have been converted into processed cheese. Keeping quality, consumer preference, packaging and storage are being studied. Though the flavour of the processed cheese made is good, the body and texture need improvement.

## Summary

The utilization of vegetable rennet from *Ficus carica* L. for the manufacture of cheddar cheese of acceptable quality has been described. The possibilities of manufacturing processed cheese of good quality from vegetable rennet cheese have also been indicated.

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# PREPARATION OF PREDIGESTED PROTEIN FOODS

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Predigested protein foods are generally prepared by enzymatic or acid-hydrolysis. In our country, attention has not been paid to the microbial digestion technique which has been extensively employed in other oriental countries for the preparation of predigested foods. The microbial process has certain advantages over other processes:

- (1) Moulds and bacteria which are employed in the preparation can be multiplied at will to digest the food materials, unlike enzymes which have to be imported and are costly.
- (2) The technique of preparing such foods is simple. In South East Asian countries, predigested protein foods such as soy sauce, *miso*, vegetable cheese, etc., are prepared in every home by microbial digestion techniques.
- (3) The biological value of the material used in the process is enhanced as it gets enriched with microbial proteins.
- (4) Foods prepared by this technique generally possess appetizing flavour.

In the Orient predigested protein foods of vegetable origin are popular since times immemorial. Soy sauce and soya bean paste or 'miso' are two such products which are liked and consumed by poor and rich alike and it is generally claimed that the secret of Chinese health lies in the consumption of large quantities of soy sauce. It has been estimated that approximately 4-6 g of high class protein is supplied in the form of soy sauce and another 2 g in the form of soya bean paste in the daily diet of the Chinese people. The chief ingredients used in the preparation of these products are soya beans, wheat or barley and sodium chloride. The extensive work carried out by earlier workers<sup>1-13</sup> has paved the way for preparing these foods on cottage and industrial scale. During the II World War, owing to the shortage of soya beans and wheat, the following materials are reported to have been utilized: soya bean meal, peanut meal, various other edible oil seed meals, silkworm pupae (defatted), wheat bran, oats, kaolings, rye and various other starchy foods. Soy sauce is also synthetically prepared<sup>7,8</sup> from a mixture of soya bean and wheat proteins by the action of hydrochloric acid, but such a preparation has poor aroma and taste to attract consumers. The mechanism involved in developing sweet aroma, taste and other characteristics in fermented products is not yet well understood.

The fermented protein foods are commonly used for seasoning vegetables, soups, gravies, sauces, sandwich spreads, salad dressing as a table relish and for the preparation of Chinese foods.

As a result of investigations carried out at this Institute, conditions have been standardised for the preparation of 'soy sauce' and similar predigested products from various protein rich materials of vegetable origin.



### Method for preparation of soy sauce

The process for the production of soy sauce developed at the Institute, is described below:

Soya beans weighing five pounds were cleaned and soaked in running water for a period of 16 hours and within that period beans were found to swell up to roughly twice the original size. The beans were then removed from water and cooked in an autoclave for a period of 2 hours at 15 lb per square inch. When this operation was in progress, cleaned wheat weighing  $2\frac{1}{2}$  lb was taken and roasted in a coffee roaster for about 15 minutes till the colour of the wheat became light brown. The roasted wheat was then coarsely ground and mixed with cooked soya beans. The mixed material was then cooled and inoculated with 150 g of Koji (1 part of rice cooked in 2 parts of tap water and *Aspergillus oryzae* strains NRRL 1988 and NRRL 1989 grown for a period of 96 hours at 28°C). The inoculated material was then spread in aluminium trays of the size 18"×14"×2½" to a depth of about 2". Each tray was then inoculated with 50 c.c. growth of *Lactobacillus delbrueckii* and 50 c.c. of *Hansenula subpelliculosa* both grown in soya bean extract medium for a period of 72 hours at 30°C (1 part of soya bean to 15 parts of water; autoclaved at 15 lb p.p. sq. in. for 20 minutes and filtered). The trays were packed in a wooden box of the size 42"×21"×18" having front and the back sides covered with fly proof galvanized mesh. The clearance space between the two trays in the box was 6". The mould growth in trays was carried out at room temperature for a period of 3-4 days till the complete mass was covered with green mould. The mouldy mass was then broken up and placed in a deep stone jars and 5 litres of 20 per cent brine solution added; The material was allowed to ripen at 37°C for a period of 2 months and the required aroma was developed. The material in the jar was stirred weekly during this period. After the ripening period, the complete mass was pressed in a wooden basket press to extract soy sauce. The residue left behind from first extraction was once again extracted after mixing it with 5 litres of hot water. The salt concentration in the second extract was raised to 18 per cent by dissolving known quantities of common salt in the extract. In a similar way, third extract was also obtained but this extract was found to be poor in aroma and other chemical constituents and as such it was used for the extraction of soy sauce from the subsequent batches. Organoleptically it was evaluated that the first and second extracts had little difference between them and hence both these extracts were mixed, heated for 30 minutes at 95°C and 0.2 g alum added. The extract was left overnight to allow the colloidal particles to coagulate and settle at the bottom of the vessel. The extract was centrifuged and clear soy sauce was then pasteurized and bottled. Each pound of soya bean gave approximately 4-5 pounds of soy sauce.

Investigations were carried out on the preparation of 'soy sauce' type of product from peanut and peanut meal. The method of preparation of sauce was same as above excepting that soya beans were replaced with peanuts or peanut meal. Soy sauce made from these materials compared favourably with the one prepared from soya beans excepting that the peanut preparation was found comparatively rich in oil as one would expect. The chemical analysis of soy sauce samples prepared from various materials is given in Table I. Experiments were also carried out to study the changes in flavour, colour, keeping quality in relation to the ratio of soya beans to wheat. The samples were evaluated organoleptically and it was found that two parts of soya beans to one part of wheat gave the best sauce,



TABLE I  
Chemical composition of soy sauce

	C.F.T.R.I.		Chinese	Japanese Noda sample
	Soy bean	Peanut		
Sp. gravity at 15°C	1.19	1.20	1.2	1.2
Total solids %	38.0	40.5	32.0	38.0
Total Ash %	20.0	19.0	...	19.7
Sodium Chloride %	18.0	17.0	16.0	18.0
Total Nitrogen %	1.63	1.4	1.0	1.5
Amino Nitrogen %	0.8	0.6	0.6	0.7
Total Acidity %	...	0.7	0.8	...
Sugars (as glucose) %	6.8	6.0	4.0	6.0
Fat %	...	5.0	...	...
pH	4.3	4.5	...	4.6
pH 10 times diluted	4.6	4.8	...	4.9

### Large scale trials in the preparation of soy sauce

Experiments were initiated in standardizing the condition for the large scale preparation of soy sauce. The method standardized for the purpose is given below:

100 pounds of cleaned soya beans were soaked in water for a period of 18 hours in a cement tank. The water was drained off and the beans packed in aluminium vessels and cooked in an autoclave for a period of 2 hours at 15 lb per sq. inch. The cooked beans were mixed with 50 lb of cracked roasted wheat. The wheat was roasted to light brown colour in a coffee roaster and coarsely powdered in a pulverising machine. The mixed material was cooled and inoculated with 3 kilograms of Koji (*Aspergillus oryzae* grown on a rice medium) and 1 litre each of *Lactobacillus delbrueckii* and *Hansenula subpelliculosa* growth obtained on soya bean extract. The inoculated material was then immediately distributed in culture trays to a depth of 2-3" placed on a movable rack. The racks were then moved in an incubating room maintaining a temperature of 26-30°C. The trays were kept there for a period of 4 days and by that time the complete mass was found covered with mould growth. The mouldy material was then broken up and transferred to wooden barrels of 100 gallon size. Approximately 120 litres of 20 per cent brine solution was added to this material. The material was allowed to ripen at room temperature (26-30°C) for a period of 3 months. The ripened material was then pressed out in a hydraulic basket press and the residue left behind was once again extracted with 120 litres of hot water. The two extracts were mixed together and enough salt added to bring its concentration to 18 per cent. In all, 220 litres of the extract were obtained. The extract was then boiled for 15 minutes in a steam double-jacketed open pan and 15 g of alum added to facilitate the settling of colloidal particles. The extract was left overnight and passed through Westfalia centrifuge to obtain a clear sauce. Soy sauce was then pasteurized at 80°C for a period of 10 minutes and enough of sodium benzoate added to give a concentration of 750 p.p.m. It was then bottled and sealed with alu-capsule. One pound of soy sauce was found to cost about twenty naye paise without a container. The storage trials of soy sauce bottles were carried out at various temperatures ranging from 25-42°C for a period of over two years. The product on keeping was found to retain the essential characteristics



TABLE II

*Chemical composition of protein rich pasty products*

						C.F.T.R.I.	Marmite	Vegemite	Bovril
Moisture %	...	...	...	...	...	41.3	28.1	36	36.7
Total ash %	...	...	...	...	...	18.8	20.2	16.0	17.1
Sodium chloride %	...	...	...	...	...	16.2	10.8	10.5	12.8
Protein (N $\times$ 6.25) %	...	...	...	...	...	28.0	41.0	33.0	39.3
Total carbohydrate %	...	...	...	...	...	10.0	...	5.0	...

of the preparation. The product was found acceptable and compared well with the reputed samples obtained from abroad.

Soy sauce is a basic raw material for the preparation of Worcestershire sauce and hence few recipes were made to suit the Indian taste. These recipes now need wide publication to attract parties interested in its manufacture. Worcestershire sauce is in great demand in our country and more so after import restrictions. The use of soy sauce in various other food preparation was investigated and its application in the preparation of savoury biscuits, sandwich making and seasoning of Indian multi-purpose food was emphasised.

Soy sauce cannot be consumed in large quantities owing to its high salt content. With an object to reduce salt content in the product and increase the protein nitrogen, experiments were conducted on the concentration of soy sauce. These efforts yielded fruitful results as it was possible to prepare a pasty product highly rich in proteins and comparatively less in its salt content. This type of product could be favourably compared in taste, aroma and other chemical constituents with Marmite, Vegemite, Bovril, etc. available in the market. The process has been covered under Indian Patent No. 64226 and released to the industry for exploitation. The product is moving well in the market under the trade name Bovite. The chemical composition of Bovite along with other products of similar type available in the market is given in Table II. In this process, enriched protein salt was obtained as a by-product. This preparation had a flavour similar to soy sauce and contained 85 per cent sodium chloride, 7.2 per cent protein (N $\times$ 6.25) and 5-7 per cent total sugars. The product could be used as a salting agent in the kitchen.

Attempts were made to produce a highly concentrated predigested protein food free of salt from soya beans and other vegetable proteins employing mould digestion technique. The experiments carried out in this direction resulted in standardizing a preparation similar to Ledinac (Lederle Laboratories Division, American Cyanamide Co., New York) having a nitrogen equivalent of 40 per cent protein, 2.1 per cent amino nitrogen, 30 per cent reducing sugars, 6 per cent total ash and sodium chloride less than 0.2 per cent. The preparation was fortified with adequate quantities of minerals and vitamins to make it a well balanced food. The techniques in the large scale preparation of this product were standardised and the complete process is now covered under Indian Patent No. 64956. Work is in



progress to assess its biological value on experimental animals. It is proposed to extend these studies to human subjects in local hospitals to determine its beneficial value as compared with similar products available in the market.

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# PRODUCTS OF PROTEIN HYDROLYSIS FROM FISH

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Fish is generally consumed either fresh or in processed condition, such as cured, dried, dehydrated, pickled or canned fish. Fish products involving proteolysis are also being developed and a few such products are already in use in some countries. The present report is discussed under three heads: (a) fish hydrolysates, (b) proteolysed fish-liver and fish-liver extracts and (c) fish peptones. All these products are protein hydrolysates derived from fish.

## Fish hydrolysates

In France, a product worked out at *Societe de Terres Rares* (S.T.R.) is based essentially on the self-digestion or autolysis of caught fish or fish wastes<sup>1</sup>. In Germany, just before and during World War II, protein hydrolysates were prepared from fish wastes. *Viking Eiweiss*<sup>2a</sup> is one such product involving chemical (alkali) hydrolysis of fish proteins. A similar product has also been reported from Norway.

The well known fish sauces of South East Asia (*Nuoc-mam* of Indo-China; *nam-pla* of Thailand; *prahoc* of Cambodia) are nothing but fish hydrolysates. van Veen<sup>2</sup> has made a review on this. Edible fermented fishery products particularly liquid preparations are practically unknown in India. Velankar<sup>3</sup> has described a method for the preparation of protein hydrolysates from fish. Using 'Ray' and 'Shark', Mahanty and Roy<sup>4</sup> worked out a method for the preparation of hydrolysed fish protein, involving alkali-digestion subsequent neutralization with acetic acid, and finally drying and defatting.

Apart from use as food or food adjuncts, fish hydrolysates can be utilized in many other ways. In Norway<sup>5</sup>, fish protein has been utilized for the manufacture of moulding powder by partially decomposing it through controlled hydrolysis which results in molecular chain length of favourable size.

Hydrolysates may be utilized for pharmaceutical purposes, as sources of specific amino acids and in this field, price is of no consideration.

Although some workers have used the method of protein hydrolysis by means of alkali, it is better to follow the method of enzymic hydrolysis particularly when it is to be used as food or for pharmaceutical purposes. Acid hydrolysis brings about loss of tryptophan and there is the problem of removal of acid. Alkali digestion brings about racemisation and loss of cystine.

## Proteolysed fish-liver and fish-liver extracts

A beginning has been made in the utilization of fish liver for the preparation of products similar to proteolysed cattle liver of commerce. In countries where the marine catch is high and where fish processing industry has developed, large amounts of fish liver is available and may be used to replace mammalian liver in the enrichment of human diet and in



the preparation of concentrates for medicinal use. Researches have been undertaken to transform liver residues from shark, cod, haddock, salmon, whale, seal, etc., into palatable forms that might be ingested as such or incorporated with other foods. Fish liver hydrolysate preparations, when successfully developed on a commercial scale will form valuable by-products to fish liver oil industries.

Guttman<sup>6</sup> carried out investigations on the preparation of extracts and enzymic hydrolysates from various fish livers and mammalian liver. The method consisted in the digestion of either whole liver or defatted liver with papain followed by pancreatin and concentrating the digest.

Liver hydrolysates or proteolysed liver are generally administered as a source of B-vitamin, particularly vitamin B<sub>12</sub> and folic acid. Tarr<sup>7</sup> has estimated vitamin B<sub>12</sub> content of fish products. Samples of different fish products were digested with papain and some with pancreatin at 37°C, for 24 hours and the hydrolysates were assayed microbiologically for substances showing vitamin B<sub>12</sub> activity. Condensed fish solubles, stick water from salmon, offal and fish liver were found to contain the vitamin. Beryl Truscott *et al.*<sup>8</sup>, worked out a method for preparing vitamin B<sub>12</sub> concentrate from cod liver residue.

Rajagopalan and Sarma<sup>9</sup> prepared extracts of shark liver residues by digestion with papain and pancreatin. Aqueous extracts have been prepared from whale livers under conditions often used for the preparation of extract for medicinal purposes<sup>10</sup>. Their vitamin and mineral composition indicates that they should be suitable alternatives to cattle liver extracts in the preparation both of pharmaceuticals and of animal feeds.

Gray *et al.*<sup>11</sup>, examined the possibility of processing hake (*Merluccius capensis*) livers whereby protein fraction and water-soluble nutritional factors may be recovered in addition to oil and oil-soluble vitamins. Methods involving partial digestion with papain and water extraction involving autolysis have been compared.

The work reviewed above suggests that fish livers so long looked upon only as potential sources of vitamins A and D, and oil, could be profitably exploited as sources of predigested protein and of B-vitamins including B<sub>12</sub> and folic acid.

### Fish bacto-peptones

This is another group of products derived by proteolytic degradation of fish. Bacto-peptones normally contain extremely variable amounts of the ill-defined products of protein-hydrolysis (proteoses-peptones-polypeptides as well as amino acids), soluble tissue extractives, B-vitamins, inorganic salts and possibly carbohydrates in small amounts. The Difco manual<sup>12</sup> gives details of analysis of their various bacto-products.

In the preparation of bacto-peptone, hydrolysis should be done with trypsin or papain. Cheldelin *et al.*<sup>13</sup>, have shown the outstanding value of papain for the liberation of seven of the more important vitamins of B group from plant and animal tissues, folic acid alone suffering slight destruction.

Vande Velde<sup>14</sup> in 1940 obtained fish peptone by neutralizing a hydrochloric acid hydrolysate of fish flesh with caustic soda and drying the solution to a powder. Tarr<sup>15</sup> in 1942 showed that tryptic digest of fish muscle formed an excellent medium for the cultivation of certain purely facultative anaerobic bacteria. Tarr *et al.*<sup>16</sup>, prepared bacto-peptones from fish flesh of marine origin with tryptic and peptic enzymes derived from



fish digestive tracts. Hydrolysis was also carried out with hydrochloric acid and caustic soda. It was observed that only those peptones which were prepared by enzymic hydrolysis consistently supported good growth of six types of *Streptococcus haemolyticus* and one *Clostridium botulinum* type E culture.

The distribution of nitrogen with respect to proteose, peptone, polypeptide and amino acids in enzymatically hydrolysed fish products was studied by Deas *et al*<sup>17</sup>. The products were prepared by digestion with fish enzymes from pyloric caeca and pancreatin for a period of 16 days.

In this laboratory, work has been in progress on the preparation of fish peptone<sup>18</sup> by papain digestion. Two different species of fish—one sea fish (mackerel—*Rastrelliger canagurta*) and one fresh water fish (*Barbus carnaticus*) are being tried.

The main object of the work at present is to work out the conditions of hydrolysis to yield maximum proportion of peptones. The muscle tissues of mackerel and *Barbus carnaticus* behaved differently in their rates of hydrolysis, as determined by the change in the amino nitrogen as percentage of total nitrogen. Rate of proteolysis was found to be somewhat greater in the case of *Barbus* sp. than that observed with mackerel. Also the distribution of nitrogen with respect to proteoses and peptones in the hydrolysates differed with the species of fish.

TABLE I

*Nitrogen distribution in different hydrolysates from fish and Difco peptone*

Description of the sample	Duration of digestion	% of total N <sub>2</sub> solubilized	Nitrogen distribution as % total nitrogen			
			Coagulable protein	Proteose	Peptone	Amino nitrogen
Hydrolysate from mackerel ...	1 hr	80.04	1.9	4.5	77.8	14.37
with papain ...	13 hr	87.24	2.1	3.0	73.2	31.27
Hydrolysate from <i>Barbus carnaticus</i> ...	2 hr	78.84	1.7	27.2	54.0	20.27
with papain ...	2 hr	79.02	0.9	24.6	61.7	25.52
Difco-peptone ...	...	...	2.5	25.2	54.5	15.6

It was observed that in both the cases, about 80 per cent of total nitrogen in the substrate was solubilized in 1-2 hours. Whereas the product from mackerel was rich in peptone and poor in proteose, product from *Barbus* was richer in proteose and poorer in peptone.

On scrutiny, it may be seen that results obtained with *Barbus carnaticus* are comparable to those of Difco peptone as carried out by us. According to the data of Difco manual<sup>12</sup> Difco peptone contains 95.17 per cent of the total nitrogen as peptone and 4.6 per cent proteose (The manual has not given any reference for the methods followed for analysis)—but following the method of Winton and Winton<sup>19</sup> we could recover only 54.5 per cent of total nitrogen as peptone and 25.21 per cent as proteose.



TABLE II  
*Difco peptone values*

		Proteose N <sub>2</sub> as % of total	Peptone N <sub>2</sub> as % total N <sub>2</sub>
As per data of Difco manual	...	4.6	95.17
As determined by us	...	25.21	54.5

Significance of the varying amounts of peptone in a preparation in relation to bacterial growth has to be studied. Another point to be investigated is the effect of non-protein nitrogenous constituents, more particularly trimethylamine oxide and urea (if present in a hydrolysate), on bacterial cultures. Investigations on this particular aspect are necessary in order that marine fish or wastes from marine fish trade or unpopular varieties of marine fish such as shark, ray, etc., could be utilized for the preparation of bacto-fish peptone. Utilization of fish trypsin and other fish enzymes for carrying out digestion requires to be investigated.

### Possibilities of developing fish hydrolysates in India

Before concluding, we may examine the prospect of developing different forms of fish hydrolysates in India. The main problem is availability of raw materials. Regarding market for finished products, there may be difficulty at the initial phase, not so much because, there is no demand for such products (e.g., proteolysed liver, liver extract, protein hydrolysate, bacto-peptone) but because people are not accustomed to such products originating from fish. This difficulty will be overcome if products obtained from fish are up to the standard.

Regarding the source of raw materials, there seems to be no dearth. To us the following seem to be potential ways for developing various forms of fish hydrolysate industry.

(1) Utilization of the total production of fish in India, is as follows<sup>20</sup>:

Total production	12 lakh tons	Consumed as fresh fish	42.7%
		Converted into sundried fish	25.9%
Fresh water fish	30%	Converted into salted fish	24.8%
Marine fish	70%	Converted into fish manure	6.6%

The fraction that is converted into fish manure can be utilized more successfully through conversion into hydrolysates.

In salting of fish, a fraction of body water comes out of the fish body. This extract also contains a certain percentage of nitrogen. While working with dry salting of mackerel we have got the following data:

Volume of water coming out per g. of dressed fish	0.25 ml
% N <sub>2</sub> in the extract	0.4%
% of total N <sub>2</sub> in fish coming out in the extract	10%

When we take into account the total quantity of fish that is salt cured, the amount of nitrogen coming out in the extract is not a negligible figure. At present, the extract and



nitrogen associated with it are wholly wasted. The main problem in utilizing the extract is its high salt content (about 25 per cent). The salt can be removed by dialysis or a fraction of nitrogen present in the extract as protein can be precipitated out by boiling.

(2) *Oil Sardine*: Catch of oil sardine in our country is quite significant. In 1957, about 191,469 metric tons of oil sardines were captured in the west coast of India<sup>21</sup>. Of course, the catch varies from year to year. Average annual catch for 1950-57 indicates that oil sardine constitutes 7.61 per cent of total marine catch. Major part of this catch is worked out for the production of sardine oil. Production of sardine oil in Madras State in the year 1954-55 was 270,000 lb<sup>22</sup>. The residue from this industry is used as guano and as fertilizer. There is ample scope for utilizing this vast residue in a better way either through its conversion into a fish flour or into some suitable form of hydrolysates. Furthermore, extraction of oil can be integrated with a process of hydrolysis of body flesh so that not only better quality hydrolytic products can be obtained, but better yield of oil will also be ensured.

(3) As with oil-sardine, residues from shark liver oil industry can be utilized for the preparation of proteolysed liver and liver extracts. In the year 1955, 26,308 gallons of shark liver oil were produced in India<sup>22</sup>. The liver residue left is not a small quantity and could be utilized in this way.

The liver residues at present are utilized mainly for the purpose of making manure. As the shark liver oil industry in our country is mostly centralised in Government hands, the scope for utilizing the liver residues or orientation of the production method so that liver forms not only the source of oil or oil-bearing vitamins, but source of proteins and B-vitamins as well, is much better.

(4) In our total marine catch, there are certain species which do not readily fetch a market. Mention may be made of Elasmobranchs (shark, ray, skate), which constitute about 3.64 per cent of our total catch<sup>21</sup>. Also, there are fish coming under the head 'Miscellaneous fish', and constituting about 4.99 per cent<sup>21</sup>. These miscellaneous fishes also fetch a low price. All these form another potential source to fall upon for developing various forms of fish hydrolysate industry to utilize our resources in a better way.

## Conclusion

We have tried to show why for better utilization of fish and fish wastes fish hydrolysate industry should be developed. Lest we be misunderstood, we should lay emphasis upon the point that fish hydrolysate industry should not be considered as a rival for fresh fish marketing or fish processing industry. Fish hydrolysate industry will utilize wastes and those sources which are not finding a good market as fresh fish or as processed fish.

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# PROTEIN HYDROLYSATES FROM INDIGENOUS SOURCES

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Imported brands of protein hydrolysates extensively used in the pharmaceutical industry either as vehicles in the compounding of liquid multi vitamin preparations or as the main constituent of the granular products employed in the oral therapy of peptic ulcer and other disorders, are of animal origin and as such, are not very popular in our country. Even assuming that such preparations can be made acceptable by effective propaganda, their production on a large scale is not feasible in view of the fact that animal proteins are at the moment in short supply in our country and their output is not expected to increase concomitantly with the needs of our growing population. It is inevitable, therefore, for us to fall back on the plant kingdom for the bulk of our protein requirements and our attention in this Institute has been directed in the last few years towards the utilization of certain agricultural or industrial waste products as sources of protein for the preparation of protein hydrolysates.

## Preparation of protein hydrolysates from oil cakes and distillery sludge, as sources of protein

We have explored the possibilities of preparing protein digests from vegetable raw material and our obvious choice was the oil seed cakes. A few attempts have been made in the past to devise processing methods for the production of a protein rich food supplement from oil cakes<sup>1</sup> or to utilize them as starting materials for the preparation of peptone<sup>2</sup>. Dark colour, off-flavour and varying degrees of the residual taste of the cakes have been the limiting factors in their general acceptance. It was felt, therefore, that if by preliminary processing, the proteins could be isolated free from the above undesirable features, they could perhaps serve as suitable starting materials adequately fulfilling the aesthetic standards of colour, smell and taste demanded of them in consumer trials. A method for preparing enzymatic digests from the proteins isolated from defatted mustard and *til* cakes has been developed in these laboratories<sup>3, 4</sup>.

Distillery sludge contains appreciable quantities of yeast and its disposal has been a problem facing the alcohol industry. Our efforts in the direction of finding a partial solution for the utilization of this waste product have resulted in the development of a process for the preparation of a palatable yeast hydrolysate from distillery sludge<sup>5, 6</sup>. Some of the properties of these two products, namely, the protein digest from sesame (*til*) and mustard cakes and the yeast hydrolysate from distillery sludge and evaluation of their quality as nutritional supplements are described in this communication.

Protein hydrolysate from oil cakes is made in three steps:

- (i) isolation of the protein from the defatted cake,
- (ii) enzymatic hydrolysis of the protein under optimal conditions and
- (iii) concentration under vacuo of the digest.



The final preparation is a granular powder, light brown in colour, readily soluble in water, slightly bitter to taste and an odour similar to that of peptone. When compounded with malt extract, chocolate, flavouring agents and other adjuvants, the taste and smell could be masked to an extent and, in limited consumer trials, oral preparations made thus were found acceptable as compared to similar preparations imported from abroad.

The process for preparing yeast hydrolysate from distillery sludge consists of:

- (i) washing the sludge free from adhering molasses and other materials,
- (ii) autolysing by pressure cooking,
- (iii) enzymatic hydrolysis of the autolysate and
- (iv) vacuum concentration and drying of the digest.

The preparation thus obtained is a brown powder, bland in taste and has a slight yeast and readily disperses in water.

The proximate composition and essential amino acid contents of the two preparations are given in Tables I and II. Composition of two imported protein digests has been included for comparison. Over sixty per cent of the nitrogen of the oil cake hydrolysate is present as amino nitrogen. Both the cake and the yeast hydrolysate contain all the essential amino acids in appreciable amounts, although the content of cystine, methionine and particularly tryptophane appear to be sub-optimal as compared to accepted standards of requirement of these nutrients<sup>7</sup>. The high content of lysine in the oil cake hydrolysate has to be stressed in view of the implicated involvement of this amino acid in the oriented synthesis of  $\gamma$ -globulin with antibody properties, collagen formation and resistance infections<sup>8-10</sup>. In addition to the above, the two preparations also contain alanine, aspartic acid, glutamic acid, threonine, serine and tyrosine. Paper chromatography revealed the presence in the oil cake hydrolysate of at least four spots corresponding to di- and tri-peptides. Significant differences were noticed in the amino acid content of yeast hydrolysate while quantitatively estimating individual amino acids in the preparation as such and after acid hydrolysis. This would mean that the yeast hydrolysate is presumably a partial digest of the yeast proteins. In addition to the essential amino acids, the yeast hydrolysate contained 72  $\mu$ g thiamin, 11.6  $\mu$ g riboflavin and 172  $\mu$ g niacin per gram. Both the preparations were rich in calcium.

TABLE I

*Proximate composition of protein hydrolysate from oil cake and distillery sludge*

					Nitrogen	Phosphorus	Ash	Amino-N
Oil cake hydrolysate	...	...	...	...	13.4	1.7	11.5	10.2
Yeast hydrolysate	...	...	...	...	10.4	0.87	7.2	...
Co-amino hydrolysate U.S.A.	...	...	...	...	11.3	...	7.6	5.8
Ledinac U.S.A.	...	...	...	...	8.0	...	2.0	1.4



TABLE II  
*Amino acid composition of protein hydrolysates  
from oil cake and sludge*

Amino acid				Oil cake hydrolysate	Yeast hydrolysate
Arginine	...	...	...	3.6	5.7
Histidine	...	...	...	2.7	2.5
Methionine	...	...	...	1.7	1.5
Tryptophane	...	...	...	0.3	0.7
Valine	...	...	...	3.5	2.5
Leucine + iso-Leucine			...	6.2	4.6
Cystine	...	...	...	1.7	...
Lysine	...	...	...	8.4	3.1
Phenylalanine	...	...	...	4.8	3.3

**Nutritive value of the protein hydrolysate**

Assessment of the biological value of the two preparations was made by a number of methods:

- (i) growth experiments on young rats receiving a synthetic diet,
- (ii) supplementation to a deficient diet,
- (iii) growth of micro organisms known to be exacting in their requirements of nitrogen and tissue culture in media constituted of the hydrolysate, and
- (iv) *in vitro* protein synthesis in a supporting media made up of the hydrolysates.

**Growth tests on rats with the oil cake hydrolysate**

The growth promoting property of the oil cake hydrolysate was evaluated by rat feeding experiments using a synthetic diet the nitrogen of which was supplied entirely by casein or the oil cake hydrolysates<sup>11</sup>. The growth data obtained during an eight week feeding period is summarised in Table III. The average gain in weight per gram of protein

TABLE III  
*Biological value of oil cake hydrolysate*

	Food intake g	Protein consumed g	Weight gain	PER g
Control (casein fed)	358	64.4	95.6	1.48
Experimental (oil cake hydrolysate)	347	62.7	85.2	1.51



consumed was not significantly different in the control and experimental animals. Presumably, supplementation of the cake hydrolysate with tryptophane would result in an enhancement of its P.E.R.

### Growth of microorganisms

Growth promoting value of the hydrolysate was also demonstrable with microorganisms known to be rather exacting in their requirement of amino acids<sup>4</sup>. Thus at a one per cent level with glucose as the energy source, the oil cake hydrolysate supported the growth of *Lactobacillus casei*, *Lactobacillus arabinosus*, *Lactobacillus fermentii* and *Lactobacillus delbruckii*. Some typical data of microbiological assays using the cake hydrolysate, Difco peptone or Ledinac are summarised in Table IV. Addition of cystine, tryptophan, methionine, or phenylalanine upto 2.0 mg/ml did not improve the growth of *Lactobacillus arabinosus* on the oil cake hydrolysate medium. Production of diastase by *Bacillus subtilis* and soil Actinomycetes is known to be significantly favoured by a complex source of nitrogen as compared to an inorganic source. In pilot shake flask fermentations using a locally isolated strain of *Bacillus subtilis* or an unidentified *Streptomyces* species, oil cake hydrolysate could replace peptone in the production of diastase (Krishna Murti, unpublished results). Similarly the oil cake hydrolysate could be substituted for peptone in the complex media routinely employed in this Institute for the production of antibiotics by soil Actinomycetes (Mrs Shete, K., unpublished work). Exploratory trials have further indicated the possibility of using the oil cake hydrolysate in place of imported lactalbumin hydrolysate in the complex aseptic medium employed for the culturing of monkey kidney cells (N. P. Gupta, personal communication).

TABLE IV

*Growth of Lactobacilli on basal media supplemented with oil cake hydrolysate, peptone or Ledinac*

Bacteria			Oil cake hydrolysate	Yeast hydrolysate	Ledinac
<i>Lactobacillus casei</i>	...	...	4.4	4.6	4.5
<i>Lactobacillus arabinosus</i>	...	...	4.4	4.8	4.6
<i>Lactobacillus fermentii</i>	...	...	2.1	4.2	3.9
<i>Lactobacillus delbruckii</i>	...	...	4.1	4.1	3.9

(Results expressed as ml 0.1 N NaOH required to neutralize acid produced in 10 ml culture in 24 hr)

### Synthesis of amylase by pigeon pancreas slices

The excretion of extracellular enzymes like amylase by pigeon pancreas is dependent upon the amino acid composition of the medium and also represents net protein synthesis by the pancreas<sup>13</sup>. The measurement of the rate of excretion of amylase by pancreas incubated in media constituted of cake hydrolysate as the sole source of nitrogen was,



therefore, employed as a basis for the evaluation of the cake hydrolysate in net protein synthesis. Results of some such typical experiments using a non-nitrogen medium supplemented with either casein or oil cake hydrolysate are summarised in Table V. It is evident from the figures that the cake hydrolysate supports *in vitro* synthesis of amylase by pigeon pancreas as efficiently as the unsupplemented casamino acids.

Supplementary value of the yeast hydrolysate to the rice diet

The nutritive value of the yeast hydrolysate was assessed by supplementation experiments on rats receiving a poor rice diet. The feeding experiment included three groups out of which one was fed the basal rice diet and the other two the basal rice diet supplemented with yeast hydrolysate at 5 and 10 per cent levels respectively<sup>44</sup>. Supplementation at these levels raised the protein contents of the basal rice diet from 8.8 to 10.6 and 13.8 respectively. The results of this preliminary experiment are summarised in Table VI. The average weekly growth of animals on the basal rice diet was 2.7 g whereas supplementation with yeast hydrolysate at 5 and 10 per cent levels raised this figure to 3.8 and 5.0 g respectively. Eight out of ten animals in the control group receiving only the basal rice

TABLE V  
*Synthesis of amylase by pigeon pancreas slices*

Incubation medium	Wt of tissue per flask (mg)	Starch hydrolysed (mg) Homogenate	Medium	Activity units
Krebs-Ringer-Bicarbonate buffer (KRB)	51	15.0	...	6.20
KRB + Casamino acids ... ..	67	11.5	33.0	13.75
KRB + oil cake hydrolysate ... ..	93	15.9	46.0	14.50

TABLE VI  
*Growth data of rats on a basal rice diet supplemented with yeast hydrolysate*

Group	Sex	Initial wt (g)	Final weight (g)		Weekly growth	% increase over control
			4 weeks	8 weeks		
Basal rice diet ...	M	21.9	34.4	...	3.1	...
	F	18.6	27.7	...	2.3	...
Basal rice diet ...	M	21.3	42.5	54.9	4.2	33.6
+ 5% yeast hydrolysate ...	F	21.0	39.0	48.5	3.4	50.0
Basal rice ...	M	20.9	47.5	66.4	5.7	84.0
diet + 10% yeast hydrolysate ...	F	21.1	47.6	62.8	5.2	127.0



diet died between the fourth and fifth weeks of the experiment. The rats on the supplemented diets were, however, comparatively healthy throughout the feeding period.

Supplementation of the basal diet by yeast hydrolysate at these levels also increased the calcium content of the diet from 0.05 to 0.08 and 0.1 per cent respectively and phosphorous content from 0.11 to 0.15 and 1.2 per cent respectively. It is likely, therefore, that although the Ca:P ratio of the diets did not substantially alter by supplementation, the stimulation of growth observed by addition of yeast hydrolysate to the basal rice diet could as well arise out of the extra calcium provided by the yeast hydrolysate. In an independent group of animals which received the basal rice diet supplemented with calcium lactate to raise the effective calcium level of the diet to 0.1 per cent, the growth stimulation observed over the control was negligible although the addition of calcium prolonged the life span of the animals which all survived the eight weeks of the experiment unlike their littermates receiving only the basal rice diet. It is apparent, therefore, that the supplementary value of the yeast hydrolysate observed in this experiment is not due to the extra calcium provided by the yeast hydrolysates. The high mortality rate and the very poor growth of the animals on the basal rice diet in the present series may be due to the low weaning weight of the rats employed. Nonetheless, this preliminary experiment has revealed the potentialities of yeast hydrolysate as a protein supplement to the poor rice diet.

Yeasts of different origin are known to cause tissue damages resembling liver necrosis and fatty infiltration<sup>15, 16</sup>. In view of this, the livers of representative animals from the three groups as well as those of some stock animals of the institute colony were subjected to histopathological examination and the observations are tabulated in Table VII. The morphological appearance of liver cells of rats receiving yeast hydrolysate supplement was comparable to normal liver cells of the stock colony. Supplementation has also conferred some beneficial effect on the rice diet in both growth stimulation as well as protection against the histological changes occurring in liver cells of animals fed only the basal rice diet.

TABLE VII

*Histopathological examination of livers of rats on basal rice diet and basal rice diet supplemented with yeast hydrolysate*

Pathological symptoms		Basal rice diet I	Basal rice diet + 5% yeast hydroly- sate II	Basal rice diet + 10% yeast hydrolysate III
Degenerative changes of parenchymal cells	...	Almost complete	Less than in I	Much less. Most of the cells normal
Plant cells	...	Present	Absent	Absent
Irregular areas of vacuolar degeneration	...	Large	Partial	Vacuoles diminishing in size. Liver cells filled with cytoplasmic material
Cellular infiltration	...	nil	nil	nil
Apparent fatty infiltration	...	nil	nil	nil
Liver capsule appearance	...	normal	normal	normal
Areas of haemorrhage	...	nil	nil	nil



Growth of microorganisms on yeasts hydrolysate

Some of the results of growth experiments using microorganisms in media made up of yeast hydrolysate are presented in Table VIII from which it is clear that the yeast hydrolysate, like the oil cake hydrolysate, is comparable to peptone in supporting the growth of bacteria.

TABLE VIII  
*Growth of microorganisms in media supplemented with yeast hydrolysate*

Medium	Vibrio cholerae	Salmonella typhosa	Escherichia coli
Nutrient broth ...	16	45	52
Nutrient broth—Peptone replaced by yeast hydrolysate (0.3%) ...	20	44	63
Yeast hydrolysate only (0.8%) ...	27	52	80
	Lactobacillus arabinosus		Lactobacillus fermentii
Micro-inoculum broth ...	49		53
Micro peptone replaced by yeast hydrolysate (0.5%) ...	41		40
Yeast hydrolysate 2.5% ...	70		40

Tubes containing 10 ml broth inoculated with a drop of washed cells of known turbidity. 18 hr growth estimated turbidimetrically (Klett, 660 mμ).

Conclusions

The results of the present study indicate that oil cakes and the alcohol distillery sludge could, by suitable processing methods, be rendered into readily assimilable forms of pre-digested proteins. The nutritive value of the preparations for laboratory animals either as the sole source of protein or as a supplement has been established. The deficiencies in the essential amino acids, particularly tryptophane and methionine, could possibly be made up by supplementation with these amino acids prepared synthetically or by fermentation. Improvement in taste and appearance and consequently ready acceptance by the consumer could be effected by addition of flavouring agents and other adjuvants.

Much remains to be done, however, before one could claim the commercial feasibility of the above processes. Thanks to the generous co-operation of the Director and staff of the Central Food Technological Research Institute, Mysore, investigations are already in progress to work out the oil cake hydrolysate process on a pilot plant scale and the preliminary trials have been encouraging. Attempts are also being made to scale up the processing operations involved in the yeast hydrolysate process in the Distillery of M/s Dyer Meakin & Co., Lucknow.



In conclusion, it is our pleasant duty to record our indebtedness to Dr B. Mukerji, Director and Dr D. L. Shrivastava, Deputy Director of this Institute, for their sustained interest in this work.

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# PROTEIN DENATURATION OF FISH TISSUE ON STORAGE IN ICE

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Fish is extremely perishable in nature. Most of the fish entering into commercial channels are preserved at about 0°C in ice with or without supplementary mechanical refrigeration. Under these conditions of storage, fish undergoes alteration, caused by autolytic enzymes, microbes and physico-chemical effects. The nature and extent of such alterations determine their storage life, consumer acceptability and price. These changes vary with the species, freshness, pre-treatment of the fish before storage and the storage conditions.

Denaturation of protein in fish tissue has been studied by many investigators<sup>1-7</sup>. Velankar and Govindan<sup>15</sup> investigated the distribution of non-protein nitrogen in a few marine fishes, crustaceans and molluscs.

Dyer<sup>8</sup> reported that sarcoplasmic or non-contractile protein showed no change in solubility on freezing except after long storage, but Mahadevan and Carter<sup>9</sup> observed that the denaturation took place with contractile protein in the frozen state. The shrinkage of muscle fibres<sup>10</sup> and denaturation of actomyosin was found to be caused by the action of salt on fish muscle. Seagran<sup>11</sup> showed that the freezing process itself produced some change in the structure of actomyosin, yielding a more symmetrical molecule without affecting its solubility, but continued storage in the frozen condition gave rise to progressive and irreversible aggregation of the actomyosin. Most of the work has been conducted with the frozen fish. But there is only very little available information on the change of protein fractions of fish tissue preserved at or near 0°C. Moreover, the work referred to above was done mainly with marine fish and a few data are available on the protein of fresh-water fish. The object of the experiments reported here was to study the changes in protein fractions of fresh-water fish, during storage in ice.

## Materials and Methods

Fresh-water fish of carp variety *Labio rohita*, was employed in the experiment. Fish was caught from a commercial 'bhari' (large catchments) and put immediately (before the onset of rigor) in ice. The temperature was maintained at 0°C throughout the experiment.

The soluble protein fractions from the ground fillets of the fish were extracted first of all with water and then with normal sodium chloride solution at 0°C. Actomyosin present in the brine extractable fractions was precipitated by the ten-fold dilution with distilled water at 4°C after standing over-night under toluene. It was then centrifuged and washed. Hamoir<sup>12</sup> showed that, under the above conditions of treatment, the extract was freed from actomyosin as he could not detect any actomyosin fraction from the electrophoretic pattern of protein extracts dialysed against a buffer of ionic strength  $\mu=0.11$  and pH=7.2.



Non-protein nitrogen was determined in the solution after precipitating the water extractable protein fractions with trichloro acetic acid. Water and brine extractable protein fractions were estimated by micro-kjeldhal<sup>13</sup> method.

The definition of myosin and actomyosin protein fractions is confusing. For this investigation, the fraction extracted by normal salt solution and subsequently precipitated by ten-fold dilution with cold water has been designated as actomyosin.

## Results and discussion

The result of the analysis of the fish tissue is given in Table I.

TABLE I  
*Chemical analysis of different protein fractions in fish tissue*

Days	Water extractable fraction (N expressed as protein) mg/g	Non-protein N-in water extractable fraction mg/g	NaCl extractable fraction (N expressed as protein) mg/g	Actomyosin mg/g	Total extractable fraction in water and brine mg/g	Percentage of actomyosin fraction of total extractable fraction
0	62.75	3.26	81.7	48.5	144.45	33.6
1	64.5	3.26	80.9	53.4	145.4	36.3
2	60.5	2.98	72.6	56.8	133.1	42.6
4	61.2	2.93	76.5	58.2	136.7	42.6
6	62.5	2.21	74.0	62.5	136.5	45.7
8	65.5	0.94	75.5	72.5	141.0	51.5
9	62.7	2.43	83.0	78.0	145.7	53.5
11	60.5	2.48	83.6	78.8	144.1	54.6
14	61.95	2.82	83.75	82.2	145.7	56.5
16	62.65	2.89	84.92	83.5	147.57	56.6
18	63.10	3.02	90.5	86.25	153.6	56.2
20	63.72	3.13	86.5	80.2	150.22	55.4
22	64.3	3.21	75.9	71.92	140.2	51.2
25	64.4	3.28	75.9	71.5	140.3	51.0
27	64.3	3.29	75.6	72.2	139.9	51.6
29	64.6	3.31	76.6	72.0	141.2	51.0
30	64.4	3.30	75.5	68.2	139.9	48.8
54	64.2	3.33	79.1	61.3	143.3	42.7
43	55.4	3.43	79.2	55.2	134.6	41.2

From the determination of the protein fractions in the fish tissue after storage for different periods in ice at 0°C, an interesting phenomenon has been observed in the case of non-protein nitrogen. The value decreases from the initial higher level, 3.26 to the minimum 0.94 within eight days of storage and after that there, is a gradual increase in the values. In support of this observation the work of Nickerson and Pactor<sup>14</sup> may be cited. They kept the Haddocks in blocks between 0°C and 17°C and observed that the values of amino nitrogen were occasionally lower than the starting level after five days, and then increased subsequently. The authors could not give any explanation of the result. The decrease in the non-protein nitrogen value may be due to either the initial bacterial attack or the activity of deaminating enzymes.

The maximum extractability with normal sodium chloride solution has been observed between 9 to 20 days of preservation after the initial fall and then there is a slight decrease during subsequent storage. It is observed from the table that the sum total of the water and sodium chloride extractable fraction does not appreciably change throughout the period of extraction and it constitutes about 75-80 per cent of the total protein.



Actomyosin percentage gradually increases from 33.6 to 56.6 within eighteen days of preservation and after that there is a sharp fall.

### Summary

Studies were carried out on the changes of the protein fractions of fresh-water fish, *Labio rohita*, during storage in ice.

Non-protein nitrogen value decreased from the initial higher level to a minimum within eight days of preservation and after that there was a gradual increase in the values. The maximum extractability with normal sodium chloride solution had been observed between 9 to 20 days of preservation after the initial fall and then there was a slight decrease during subsequent storage. The sum total of the water and the sodium chloride extractable fraction did not appreciably change during the period of storage.

Actomyosin percentage gradually increased to a maximum and after that it began to decrease sharply.

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# EFFECT OF PROCESSING ON THE SOLUBILITY OF PROTEINS IN SOME STRAINED BABY FOODS

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In the course of investigations on the development of strained baby foods based on some of the typical Indian fruits and vegetables, some products were developed in which skimmed milk powder formed one of the ingredients in the recipe. Further, in addition to canning, the products standardized were dried on a roller-drier to facilitate their storage and distribution to average Indian homes. The heat processes involved in canning as well as drying are likely to affect the solubility of the proteins present in these products. It was, therefore, of interest to study the effect of the various processes involved on the solubility of the proteins. The results are presented in this paper.

Rice and Beuk<sup>1</sup> have reviewed the effects of heat processing on the nutritive value of proteins. Joly<sup>2</sup> has discussed the reversible denaturation of proteins. The amino acids of pure proteins are quite stable towards heat. In mixtures, however, particularly in the presence of non-protein materials like carbohydrates, they may not be equally stable. Woods, Beeson and Bolin<sup>3</sup> found that heat treatment reduced the protein value of field peas. Armbruster and Murray<sup>4</sup> reported that protein quality of peas was not impaired on canning. The experimental results regarding the effect of heat on protein quality have not so far been conclusive<sup>1</sup>. Joly<sup>2</sup> states that it has not so far been possible to give a quantitative structural description of heat-denaturation of proteins under any given conditions. Generally, the insolubility of the protein in a medium in which it is soluble, while still native, is taken as the criterion for determining its denaturation<sup>5</sup>. In the present investigation, loss of solubility in water and salt solution has been adopted as the main criterion.

## Materials

Banana and mango custard blends and strained pea and field bean pulps were employed in this investigation. These products were prepared by the methods standardized in these laboratories<sup>6,7</sup>. These were briefly as follows:

*Banana custard blend and powder:* Ripe *Pachabale* banana was used. The pulp was steamed for 10 minutes and pulped through a 40 mesh sieve. To the pulp (3,200g), skimmed milk powder (160 g), sugar (160 g) and corn starch (80 g), were added and the blend heated in a steam-jacketed kettle to gelatinize the starch. The acidity of the blend was reduced to 0.25 per cent by partial neutralization with sodium bicarbonate, and the blend then homogenized. One portion of the blend was canned by filling it hot into cans of size 300×308, sealing them and processing for 30 minutes at 240°F. The remaining portion was dried in a laboratory double drum drier at atmospheric pressure under conditions already described in a previous communication<sup>7</sup>. The dried product was powdered and preserved in air-tight tin containers.



*Mango custard blend and powder:* Badami mango pulp, fresh as well as cold-stored at 35°F, was used in the preparation. The method of preparation was similar to that in the case of banana products, except that followed in one case, the acidity was adjusted in the pulp and in the other case in the cooked custard blend.

*Green peas:* Fresh and tender garden peas were shelled by hand. The peas were covered with the minimum quantity of water and autoclaved for 15 minutes at 10 psig to render them soft. They were then pulped and strained through a 60-mesh sieve to remove the coarse fibre. One portion of the strained pulp was canned in plain cans by adopting a process of 60 minutes at 240°F. Aliquots of the rest of the pulp were dried in (i) an atmospheric double drum drier, (ii) a cross-flow cabinet drier at 140°F and (iii) a vacuum-shelf drier under 25 inches of vacuum. The dried materials was powdered and kept in air-tight tin containers.

*Field beans:* Canned and dried products were prepared from the tender and green field beans by adopting methods similar to those in the case of green peas.

### Analytical methods

*Sampling:* The wet material was ground to a pulp of uniform consistency. The dried powders were ground in a laboratory model Wiley mill using a 40-mesh sieve. Representative samples were drawn for analysis from the prepared material.

*Moisture:* This was determined by drying the material in an air oven to constant weight.

*Water soluble protein:* A weighed amount (5.0 g) was made to 100 ml with distilled water and the mixture shaken in a mechanical shaker for 1 hour. The mixture was kept overnight in a refrigerator at 42°F, filtered through Whatman no. 1 paper and an aliquot of the filtrate taken for the determination of nitrogen.

*Salt soluble protein:* A 5.0 per cent sodium chloride solution was used for the extraction.

*Total protein:* This was determined by micro-Kjeldahl method<sup>8</sup>.

*pH:* The pH of the blends was determined by means of a Beckman pH meter using glass electrode.

### Results and Discussion

Data regarding total, water soluble and salt soluble proteins at different stages of processing of banana custard product are given in Table I. It will be seen that there is generally a gradual decrease in water and salt solubility of proteins in the successive stages of processing, the salt solubility of the proteins being slightly greater than their water solubility.

Of the three ingredients—starch, sugar and skimmed milk powder—used in the preparation of the custard blend, only the skimmed milk powder contains protein and this accounts for the increase in the total protein content from 1.10 per cent to 2.05 per cent in the custard blend. The skimmed milk powder contains 32.8 per cent total proteins of



TABLE I

*Effect of different processes on the water and salt solubility of proteins  
in banana custard blend*

Material		On fresh weight basis		
		Total protein (N×6.25) %	Water soluble protein	Salt soluble protein
			as per cent of total protein %	as per cent of total protein %
Raw fruit	...	1.10	39.34	53.43
Fruit after steaming	...	0.94	49.78	39.27
Raw strained custard blend	...	1.93	68.41*	75.78*
			34.94†	36.13†
Cooked custard blend	...	2.05	21.95	34.12
Canned custard blend	...	2.05	24.01	24.20
Cooked custard blend, drum dried	...	7.32	24.44	25.85
Canned custard blend, drum dried	...	7.27	15.71	22.21
Skimmed milk powder	...	32.80	92.87	96.70

\* Expected value. † Actual value.

which 92.8 per cent is water soluble and 96.7 per cent salt soluble. The solubility of the protein decreases considerably even on blending the ingredients. Cooking of the custard blend prior to canning results in a further decrease of the water solubility of the proteins from 35 to 22 per cent and of salt solubility from 36 to 34 per cent. On canning, there is a further decrease in salt solubility to 26 per cent. Drum drying does not lead to any further significant decrease in this solubility. This might be due to the fact that in drum drying, the material is subjected to a high temperature of about 350°F, for a short period of 20 to 30 seconds only. In canning, however, the heating is comparatively more drastic, as the canned product is processed for as long as 30 minutes at 240°F.

*Mango custard:* In the case of mango custard blend, data regarding the effect of (i) partial neutralization of the acidity of the pulp prior to addition of the other ingredients and (ii) of the custard blend after cooking on the solubility of protein are given in Table II. The results are almost similar to those of the banana custard blend. In the case of both the treatments, there is a decrease in the solubility of protein in water as well as in salt solution as a result of heating during cooking and canning. The loss in solubility is comparatively slightly less in drum drying than in canning of the custard blend and this is accounted for by the obvious differences in the degree of heating involved. Partial neutralization of the custard mix at the end of cooking resulted in higher water and salt solubility of the protein in the dried powder than partial neutralization at start prior to mixing the other ingredients (19 per cent and 40 per cent as against 12 per cent and 22 per cent respectively). The former procedure, therefore, is to be preferred in the preparation of mango custard powder.

*Field beans and green peas:* Tables III and IV give the solubility data of field bean and green pea pulps. Initial autoclaving of the beans results in a decrease of the water solubility of the proteins from 57.0 per cent to 18.6 per cent and of salt solubility from



TABLE II  
Effect of partial neutralisation of acidity of mango pulp on water and salt solubilities of protein in mango custard blend

Acidity adjusted after cooking the custard mix														
Acidity of pulp adjusted at start					Acidity adjusted after cooking the custard mix									
Particulars	pH	% Moisture	On moisture free basis				pH	% Moisture	On moisture free basis					
			Total protein (N × 6.25) %	Water soluble protein (N × 6.25) %	Salt soluble protein (N × 6.25) %	S.S.P. × 100 T.P. %			Total protein (N × 6.25) %	Water soluble protein (N × 6.25) %	Salt soluble protein (N × 6.25) %	S.S.P. × 100 T.P. %		
A Pulp ...	3.7	81.75	6.00	2.57	2.21	42.77	36.75	3.7	81.75	6.00	2.57	2.21	42.77	36.75
A <sub>1</sub> Pulp in which the acidity was adjusted before mixing starch sugar and milk powder	4.9	77.64	11.32	7.51 1.87	7.35 5.39	66.33 16.52	64.75 (a) 47.56 (b)	4.2	77.64	10.58	6.82 1.60	6.64 1.78	64.51 15.10	62.76 (a) 16.79 (b)
A <sub>2</sub> Cooked custard blend ...	4.9	77.00	11.15	3.62	2.34	32.48	20.94	5.0	77.00	10.69	2.07	2.81	19.32	26.22
A <sub>3</sub> Canned custard blend ...	4.9	77.45	11.03	1.48	1.84	13.42	16.70	4.9	76.95	10.43	1.79	1.72	17.77	16.44
A <sub>4</sub> Drum-dried custard powder	4.9	1.78	11.18	1.39	2.50	12.41	22.35	5.0	1.91	10.96	2.06	4.31	18.79	39.78
					On moisture free basis									
					Total protein (N × 6.25) %	Water soluble protein (N × 6.25) %	Salt soluble protein (N × 6.25) %	S.S.P. × 100 T.P. %						
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					Total protein (N × 6.25) %	Water soluble protein (N × 6.25) %	Salt soluble protein (N × 6.25) %	S.S.P. × 100 T.P. %						
					6.00	2.57	2.21	42.77						



TABLE III  
*Effect of different treatments and processes on water and salt solubility of proteins in field beans*

Treatment	Total protein (N $\times$ 6.25)	On fresh weight basis	
		Water soluble protein	Salt soluble protein
		as per cent of total protein %	as per cent of total protein %
Fresh beans	10.46	56.98	69.23
Autoclaved field beans	7.31	18.57	19.89
Covering liquid	1.15	...	...
Strained pulp	5.11	21.48	23.47
Coarse residue	4.44	23.34	23.47
Strained pulp, canned (from 4)	5.36	17.65	20.89
Strained pulp, (from 4) drum dried	29.42	18.46	19.07
Canned pulp, (from 6), drum dried	29.31	18.85	20.01
Strained pulp (from 4), dried in a cabinet drier	29.70	13.67	15.81
Strained pulp (from 4), dried in a vacuum-shelf drier	29.69	12.30	13.95

TABLE IV  
*Total, water and salt soluble proteins present in drum-dried strained pea pulp powder*

	Quantity present in material (N $\times$ 6.25) %	As per cent of total protein %
Total proteins ...	24.53	...
Water soluble protein ...	9.48	38.65
Salt soluble protein ...	10.25	41.78

69.2 per cent to 19.9 per cent. The subsequent processes of heating of pulp, canning and drum drying do not lead to any further decrease in their solubilities. Cabinet drying and vacuum-shelf drying, however, slightly lower the solubility on account of the comparatively longer period of heating involved.

In the case of drum-dried pea pulp powder, which contains 24.5 per cent of total proteins, the solubility of the protein is much higher than in the case of the field beans (39-42 per cent). It is yet to be seen to what extent these reductions in the solubility of the proteins affect the actual nutritive and biological value of the products. The observations of Greaves, Morgan and Loveen<sup>9</sup> that heating of casein for 8 hours at 100°C (212°F) did not reduce the growth-promoting properties and of Mabee and Morgan<sup>10</sup> that even heating for 15 minutes at 140°C (280°F) had very little effect on the growth-promoting



index of casein are of particular interest in this context. It is proposed to investigate this aspect of the problem by carrying out actual rat feeding experiments.

### Summary and conclusions

In the preparation of strained baby foods either by canning or by drum-drying, there is a considerable decrease in the water and salt solubility of proteins. In fruit custard blends, which contain added skimmed milk powder as one of the ingredients, the solubility of the milk proteins considerably decreases even on blending of the ingredients. On the other hand, partial neutralization of the acidity in the custard mix at the end of cooking is preferable, as it results in a higher solubility of the proteins in the dried product than initial neutralization in the pulp. In the case of peas and field beans, considerable loss in the solubility of the native proteins occurs even in the initial process of autoclaving to render them soft. In the custard blends as well as the vegetable pulps, there is a gradual loss in the solubility of the proteins in the successive stages of processing. The solubility of the proteins in the drum-dried product is generally slightly higher or of about the same order as that in the case of the canned product. This loss in solubility of protein may not, however, affect the actual nutritive and biological value of the product to the same extent. This requires to be confirmed by actual feeding experiments.

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# SOME OBSERVATIONS ON THE PREPARATION OF MEAT EXTRACT, MEAT SOUP CUBES AND CHICKEN ESSENCE

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## Meat extract

Commercial meat extract is, at present, prepared by concentrating the small fraction of meat solids extracted during scalding of meat before curing for the preparation of corned beef<sup>1</sup>. When meat is solely processed for the preparation of meat extract, it is minced and scalded in boiling water and the extract is concentrated. In normal culinary practices, as in the preparation of broth, meat is simmered for lengths of time exceeding one and half hours. Hence, it is of interest to follow progressive extraction of meat solids.

Meat from animals slaughtered and dressed the previous evening and hung overnight in a well-ventilated place was obtained in the morning from the local market. The leg of mutton (sheep) was cleaned of all surface fat, deboned and minced. An aliquot of the minced meat was analysed for total nitrogen, ash, fat, chloride, total creatinine and reducing sugars<sup>2</sup> (Table I). 200 g aliquots of the minced meat were boiled with 800 ml of water under reflux for 15, 30, 45 and 60 minutes. At the end of the extraction, it was filtered. Solids, total nitrogen, ash, chloride, total creatinine and reducing sugars were estimated in the filtrate (Table II and III). Analysis of solids obtained by repeated extraction of minced meat at room temperature to remove all the soluble constituents has also been included in Tables II and III. The residue left after the extraction was hydrolysed using papain and utilized for preparation of soup cubes.

From Tables II and III, it is apparent that the leaching of meat solids is not increased with boiling the minced meat with water for times longer than 30 minutes. Only a quarter of the total creatinine and one-eighth of the reducing sugars present in the original minced meat are extractable. This may be due to the retention of a part of the extract

TABLE I

*Analysis of minced meat \* (as percentage of solids)*

Constituent		Fresh basis	Dry basis
Total nitrogen	...	3.190	11.36
Total protein	...	19.94	70.99
Ash	...	1.025	3.649
Fat	...	6.488	23.10
Cl' as NaCl	...	0.1880	0.6692
Total creatinine	...	0.6080	2.164
Reducing sugars	...	0.5216	1.857

\* The moisture content of the minced meat used was 71.91%.



TABLE II

*Analysis of meat extracts prepared at room temperature and by boiling minced meat\*  
(as percentage of different constituents of meat)*

Constituent	Cold extract	Boiling extract			
		15 minutes	30 minutes	45 minutes	60 minutes
Solids extracted ...	21.3 (21.0—22.0)	9.6 (9.1—9.7)	10.4 (10.0—10.8)	9.9 (9.7—10.5)	9.9 (9.8—10.5)
Total nitrogen ...	16.2 (16.1—16.5)	8.9 (8.6—9.7)	8.9 (8.4—10.1)	8.7 (8.2—9.8)	8.9 (8.6—9.4)
Ash ...	32.7 (32.1—32.9)	75 (69—77)	77 (75—78)	78 (75—79)	78 (77—79)
Cl' as NaCl ...	37.6 (37.1—39.2)	87 (85—92)	87 (86—90)	85 (83—86)	88 (87—89)
Fat ...	...	0.25	0.31	0.25	0.31
Total creatinine ...	100	26.5 (25.0—28.0)	27.9 (26.9—28.3)	26.8 (25.3—27.5)	26.9 (26.4—27.7)
Reducing sugars ...	100	11.5 (11.2—12.0)	12.3 (12.2—12.6)	12.7 (12.5—12.9)	12.5 (12.3—12.7)

\* Values are average of six batches. Figures in parenthesis indicate ranges.

TABLE III

*Analysis of meat extracts prepared at room temperature and by boiling minced meat\* (expressed as mg./ml.)*

Constituents	Cold extract	Boiling extract			
		15 minutes	30 minutes	45 minutes	60 minutes
Solids extracted ...	15 (14.8 —15.5)	6.8 (6.4 —6.9)	7.3 (7.0 —7.6)	7.0 (6.9 —7.4)	7.0 (6.9 —7.4)
Total nitrogen ...	1.3 (1.3 — 1.4)	0.71 (0.69—0.78)	0.71 (0.68—0.80)	0.70 (0.65—0.79)	0.71 (0.69—0.75)
Ash ...	0.84 (0.83— 0.85)	1.9 (1.8 —2.0)	2.0 (1.9 —2.0)	2.0 (1.9 —2.0)	2.0
Cl' as NaCl ...	6.18	0.41 (0.40—0.44)	0.40 (0.40—0.43)	0.40 (0.39—0.40)	0.41 (0.41—0.43)
Fat ...	...	0.04	0.05	0.04	0.05
Total creatinine ...	1.5	0.40 (0.38—0.43)	0.43 (0.41—0.43)	0.64 (0.60—0.65)	0.64 (0.64—0.66)
Reducing sugars ...	1.3	0.15 (0.15—0.16)	0.16	0.16	0.16

\* Values are average of six batches. Figures in parenthesis indicate ranges.



in the residue during filtration as also to some binding of the reducing sugars with free amino groups to form Maillard compounds. Extraction of minced meat with water at room temperature removes 20 per cent of meat solids. Such extract cannot be resorted to for preparation of meat extract since a large part of the protein solubilized gets precipitated during concentration by heat coagulation.

There are no official standards for meat extract. According to the definition suggested by the Association of Official Agricultural Chemists,<sup>3</sup> meat extract is the product obtained by extracting fresh meat with boiling water and concentrating the liquid by evaporation after removal of fat. It contains at least 75 per cent of total solids of which, not more than 27 per cent is ash and not over 17 per cent sodium chloride. The fat should not exceed 0.6 per cent and the nitrogen not less than 8 per cent and total creatinine not less than 10 per cent.

The analysis of concentrated meat extract is presented in Table IV. This analysis conforms to the tentative standards suggested by A.O.A.C. Only in the case of total creatinine, the values obtained in this analysis are much lower than the value suggested. This point is now under study.

TABLE IV  
*Analysis of meat extracts\* (as percentage of solids)*

Constituents		Extract of minced meat by boiling			
		15 minutes	30 minutes	45 minutes	60 minutes
Total nitrogen	...	10.10	10.94	10.53	10.17
Total protein	...	63.16	68.34	65.83	63.59
Ash	...	29.03	29.71	29.60	29.69
Fat	...	0.60	0.63	0.59	0.65
Cl' as NaCl	...	5.879	5.782	5.919	5.954
Total creatinine	...	6.566	5.670	5.671	5.738
Reducing sugars	...	2.28	2.11	2.23	2.23

\* The extracts analysed were obtained by concentrating the hot extract to about 75 to 80% total solids.

In Industry, four to five batches of meat cubes are scalded in the same boiling water before it is concentrated to the meat extract of commerce. The greater proportion of creatinine in this extract may be due to the comparatively higher leaching out of creatinine than the other soluble constituents during repeated use of the same scald water.

Meat soup cubes

Commercial bouillon cubes contain beef extract, protein hydrolysate, gelatin, spices and salt. Generally, vacuum shelf drying is resorted to, for drying the base<sup>4</sup>.



Preparation of meat soup cubes (mutton-sheep) has been undertaken here, using meat extract, meat hydrolysate, hydrogenated fat, pepper extract, gelatin and salt.

### Technique of hydrolysis

Before hydrolysis, the meat is minced and extracted in four times the amount of water by boiling for 30 minutes. The broth is filtered off and the residue is taken up for hydrolysis.

(i) Papain hydrolysis was carried out at pH 4.5 and 55°C, for 16-18 hours. 3 g of papain was used per pound of boiled minced meat suspended in two pounds of water. Chloroform and toulene were used as antiseptics. After the incubation, the pH of the suspension was adjusted to neutral, then boiled and filtered. The amount of nitrogen brought into solution in the various trials was on an average, 85 per cent. The residue left after the filtration was added to the next lot of meat to be hydrolysed.

(ii) Acid (HCl) and alkali (NaOH) hydrolysis were also carried out at 15 lb pressure for 2 hours. The quantity of hydrolysing agent (2N) used was double the quantity of substrate. After the pressure heating, the hydrolysates were cooled and neutralised. Any precipitate was filtered off. This type of hydrolysis brought all the nitrogen in the meat into solution.

For neutralization, mixing of equivalent amounts of acid and alkaline hydrolysate has been tried. Removal of hydrochloric acid from the hydrolysate by vacuum distillation has also been carried out too.

Papain hydrolysis gives a straw-yellow coloured hydrolysate, bitter in taste. It has been shown by Murray and Baker<sup>5</sup> that the bitter flavours are not from the enzymes themselves, that the treatment of the hydrolysates with activated carbon substantially improves their taste, but removes much of the tryptophan as well. This improvement to a bland and meaty flavour is mainly due to the removal of polypeptides by adsorption on charcoal.

Acid or alkali hydrolysis gives a hydrolysate bland with acceptable colour. These two characteristics are desirable in a soup base.

### Drying of the hydrolysate

When the hydrolysates are ready, they are blended with the broth removed before hydrolysis of the meat, calculated amounts of gelatin are added and dissolved and the whole concentrated to about 70 per cent solids.

Drying of the soup bases was carried out in the vacuum shelf drier at 50-60°C, and 27" vacuum and in the freeze-drier. In freeze-drying, cycle was started after the soup base was frozen and finished off at 50-60°C. During the whole period of freeze drying, a vacuum of 27" was maintained. After drying, the necessary amounts of salt, pepper extract and hydrogenated oil are suitably mixed with the soup base and the whole mix allowed to mature for two to three days in airtight containers.

The approximate composition of the soup mix is as follows:

Meat solids	45 per cent
Salt	45 per cent
Fat	5 per cent
Pepper extract	2 per cent
Gelatin	3 per cent



Increasing the proportion of meat solids to more than 45 per cent at the cost of salt imparts a bitter taste of the hydrolysate to the final product.

The analysis of a representative batch of freeze-dried soup mix is given in Table V.

TABLE V  
*Analysis of free soup mix*

Constituent	I Acid hydrolysate + NaOH %	II NaOH hydro- lysate + HCl %	III Acid hydrolysate + Alkali hydrolysate %	IV Acid hydrolysate- acid removed by evaporation %
Moisture ...	5.59	4.87	4.81	8.44
Nitrogen ...	6.34	6.08	6.55	5.98
Protein ...	39.64	38.01	40.97	37.36
Fat ...	6.04	5.52	5.18	5.71
Ash ...	44.88	48.07	46.38	42.30
Acid insoluble ash ...	0.14	0.11	0.38	0.46
NaCl ...	39.38	35.50	37.31	37.70
Total creatinine ...	0.65	0.53	0.41	0.54
Organic matter ...	49.53	43.06	48.81	49.26

Sample No. III was prepared by mixing equivalent amounts of acid hydrolysate and alkaline hydrolysate and the requisite quantity of salt was added. Acid or alkali of 2N strength was used for hydrolysis, because, on neutralizing, samples I and II provide the correct proportion of sodium chloride and meat solids. In the case of sample No. IV, the required quantity of salt has to be added.

Studies on the vacuum shelf-dried products are in progress.

In a product of this type which is 'ready-to-serve', packaging of unit tablets assumes importance. Similar product in the form of cubes of 1 cm dimension is imported into India and the product has been quite popular. Since the product made in these studies have to be used in single unit so as to be enough for one serving, the material was pressed into 1 cm. cubes weighing about 4.5 g. The sample as made and equilibrated to 50 per cent R.H. had a moisture content of 5.5 per cent. Packaging characteristics of this product were determined and it was found that, the critical moisture content was about 10 per cent from the point of mould growth while the product shows signs of becoming soft at about 8 per cent. It therefore, appears that the product does require a fairly good moisture barrier so that the packed product put into bottle could have a shelf life of more than 12 months. Work on the suitability of variety of packaging films like moisture-proof cellulose film and aluminium foil with an inner layer of grease-proof paper is in progress.

### Essence of chicken

Though no definition of the term 'essence of chicken' is available, analogy may be drawn from a formal definition<sup>6</sup> of 'essence of beef' which is termed as the product



obtained by the extraction of minced beef with boiling water, such extraction being sufficiently prolonged to produce a jelly on cooling. The commercial 'essence of chicken' seems to be hydrolysate of chicken meat, possibly also mixed with a boiled water extract.

The value of boiled extract of chicken meat rests on two factors, viz., (i) as a stimulant for gastric secretion on account of its flavour and presence of nitrogenous extractives; and (ii) the presence of water-soluble nutrients leached out into the extract. A hydrolysate has the advantage of being a source of amino acids and peptides for building of body tissues.

When much dependence is placed on the hydrolysates as source of protein for therapeutic purposes, it is necessary to ensure adequate quantities of vitamins and minerals that are essential for protein metabolism. Deuel<sup>7</sup> has discussed the indispensability of the vitamins of the B-complex in protein metabolism. Minerals such as phosphorus, sulphur and potassium are necessary for the incorporation of nitrogen in the protoplasm<sup>8</sup>. It is therefore, essential to retain the nutrients available in chicken meat in the product.

The present work has been undertaken, in response to requests from the Industry, to evolve a standard product. Results of analysis of market samples of essence of chicken

TABLE VI  
*Analysis of essence of chicken (market samples)*

Sample No.	Total solids %	Nitrogen %	Protein (N × 6.25) %	Total creatinine %	Ash %	Chloride as NaCl %
I	6.69	0.38	2.38	0.14	0.93	0.46
II	16.48	2.38	14.88	0.29	1.21	0.68
III	8.61	0.42	2.63	0.26	1.17	0.51
IV	11.04	1.35	8.44	0.21	1.08	0.54
V	6.58	0.92	5.75	0.20	0.91	0.48

for total solids, nitrogen, total creatinine, ash and chloride, are presented in Table V. The samples were in 10 ml. ampoules.

It may be observed from the above data that the total solids and nitrogen content of the market samples vary. Since the pharmaceutical value of these products, apart from natural or added vitamins and minerals depends upon their nitrogen (protein) content, laying down of minimum requirement for nitrogen content seems very necessary.

Total solids and nitrogen contents of three of our own preparations derived from chicken meat were as follows;



It may be seen that the total solids are nearly accounted for by the protein material ( $N \times 6.25$ ). Among the commercial samples, (ii) and (v) are on par with our samples, in this respect.

TABLE VII  
*Analysis of chicken meat preparation made by the authors*

Samples	Total solids %	Nitrogen %	Protein ( $N \times 6.25$ ) %
Pressure-cooked extract of chicken meat ...	3.0	0.42	2.63
Papain hydrolysate obtained from meat residue in 1 ...	13.5	1.95	12.19
Papain hydrolysate of whole chicken meat ...	14.7	2.19	13.69

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## IV. NUTRITION

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# NUTRITIVE EVALUATION OF VEGETABLE PROTEINS

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Several investigators have determined the nutritive value of the proteins of cereals and pulses. Mention may be made of Swaminathan<sup>1</sup> and Basu *et al.*,<sup>2</sup> among others, for, they did the pioneering work to initiate the researches in this field. The information gathered from these investigations is summarised in Table I.

TABLE I  
*Biological value, digestibility coefficient and growth efficiency of common Indian dietary proteins*

Foodstuff	Digestibility coefficient per cent	Biological value per cent	Growth efficiency Ratio (8 weeks)
<b>Cereals*</b>			
Rice ... ..	96	80	1.7
Wheat...	93	66	1.3
Jowar...	91	83	0.8
Ragi ...	79	89	0.7
Maize...	80	60	1.0
Bajra ...	89	83	1.1
Italian millet ...	91	77	...
<b>Pulses</b>			
Red gram ... ..	75	72	0.7
Bengal gram ...	82	74	1.1
Green gram ...	85	54	0.8
Black gram ...	80	63	1.0
Lentil ...	85	49	0.5
Peas ...	90	59	1.1
Cow pea ...	78	45	...
Field bean ...	70	49	...
Soya bean ...	90	60	0.9

\* At 5 per cent and the rest at 10 per cent level of protein in the diet.

The biological value of cereal proteins varies between 60 to 89 per cent and those of legumes between 45 and 74 per cent. The biological value of legume proteins is lower than that of cereals and the digestibility coefficients also show the same trend. As far as the protein efficiency ratios are concerned, both cereal and pulse proteins have lower values as compared to animal proteins and are inadequate for promoting optimal growth.

## Essential amino acid composition

The classical work of Rose<sup>4</sup> has left little doubt now that protein nutrition is essentially amino acid nutrition. Block and Mitchell<sup>5</sup> brought forward the evidence that



the nutritive value of a protein depended to a large extent on its essential amino acid composition. Many of the vegetable proteins have been analysed by Indian workers for their essential amino acid composition. Balasubramanian *et al.*,<sup>6,7</sup> Vijayaraghavan and Srinivasan<sup>8</sup> and Ramachandran and Phansalkar<sup>9</sup> can be particularly mentioned among the workers in this field, for, between them they have covered cereals, pulses, tubers and green leafy vegetables. The survey of essential amino acid composition reveals that as compared to whole egg protein, cereals are deficient in lysine, pulses in methionine and tryptophan, tubers and green leafy vegetables in the sulphur amino acids. The easiest way to improve the nutritive quality of a protein is by supplementation with the deficient amino acids. This, however, does not appear to be practicable on a large scale in the realm of human nutrition. It is far more practical to improve the nutritive value of cereal proteins by incorporating in the diet other foodstuffs which will supply the deficient amino acids.

### Nutritive value of mixed vegetable proteins

Swaminathan<sup>1</sup> did not observe any supplementary effect between cereal and pulse proteins in 1:1 proportion by the nitrogen balance method. The diet surveys done in India show that cereal and pulse proteins are more likely to be consumed in a proportion of 2:1 than 1:1 and hence the determination of nutritive value in this proportion was considered to be of more practical significance. Infants in post-weaning period and growing children, are given practically the same type of diets as those taken by adults in India. The pressing problem of protein malnutrition and its prevalence in infants and children made it necessary to determine the efficacy of vegetable proteins for promoting growth and to find out better ways to supplement the available resources of vegetable proteins.

Phansalkar *et al.*,<sup>11</sup> therefore, determined the protein efficiency ratio (PER) of cereal proteins singly and in admixture with pulse proteins. The effect of supplementation of dry leafy vegetable protein was also studied in some cases. The experiments were carried out with young weanling rats from the stock colony and protein efficiency ratio was determined by feeding a number of cereal, pulse and cereal-pulse combination diets for a period of 28 days.

The proportion of protein in the different diets was as follows: 1.10 per cent cereal or pulse protein; 2.7 per cent cereal protein; 3 per cent pulse protein; and 3.6 per cent cereal protein+3 per cent pulse protein+1 per cent leaf protein.

The comparison of the PER was also made with 10 per cent skim milk protein diet. The results of these experiments are depicted in Figure 1 and Table II.

The results indicated that in most cases mixtures of cereal and pulse proteins in the proportion 7:3 had significantly higher PER than when cereal or pulse formed the sole source of protein. The incorporation of leaf protein yielded a protein mixture which approached skim milk in PER.

The haemoglobin and plasma protein levels were studied at the end of the fourth week in the same animals. It was observed that on the cereal diet alone these levels were lower than in the animals on the stock diet, rice diet being the only exception. The pulses alone, on the other hand, produced significantly higher levels than the cereals.



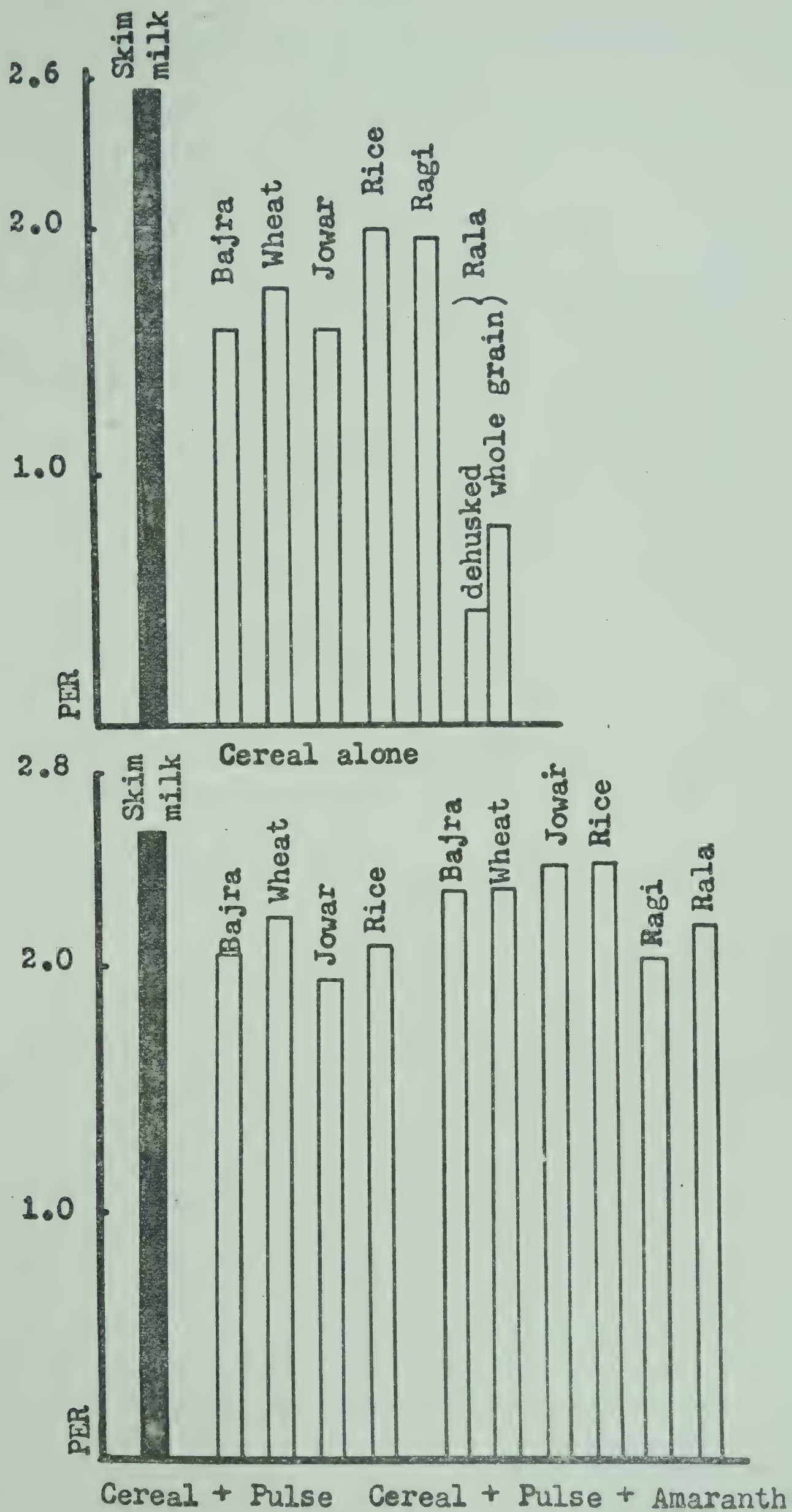


FIGURE 1. Improvement in Protein Efficiency Ratio (PER) of different cereals on supplementation (Period—28 days).



TABLE II  
*PER of cereals alone and after supplementation with a pulse  
 and leafy vegetable*  
 (Level of protein intake % ; duration 28 days)

Protein source in diet	PER
Rice ... ..	2.0
Rice + Red gram + Amaranth ... ..	2.2
Wheat ... ..	1.8
Wheat + Red gram + Amaranth ... ..	2.3
Bajra ... ..	1.6
Bajra + Red gram + Amaranth ... ..	2.2
Jowar (with husk) ... ..	1.4
Jowar (dehusked) ... ..	0.9
Jowar + Red gram + Amaranth ... ..	2.4
Ragi ... ..	2.0
Ragi + Bengal gram + Amaranth ... ..	2.1
Samai ... ..	1.1
Samai + Bengal gram + Amaranth ... ..	1.8
Italian millet (tenai) with husk ... ..	0.8
Italian millet (without husk) ... ..	0.5
Italian millet + Bengal gram + Amaranth ... ..	2.2
Kodra (varagu) ... ..	0.7
Kodra + Bengal gram + Amaranth ... ..	1.9
Rajkeera ... ..	1.8
Rajkeera + Bengal gram + Amaranth ... ..	2.0
Coconut cake ... ..	1.4
Bajra + coconut cake (6: 4) ... ..	1.6
Bajra + coconut + redgram (6: 2: 2) ... ..	2.0

The cereal plus pulse combination brought about significant rise of haemoglobin in the case of wheat and jowar and in plasma protein in the case of wheat, jowar and bajra.

### Haemoglobin and plasma protein regeneration

The results mentioned above make it obvious, therefore, that suitable vegetable protein mixtures bring about growth almost equal to that of skim milk protein. Whether they would be equally effective in regenerating haemoglobin and plasma proteins in severely protein depleted animals was further studied by Phansalkar *et al.*<sup>10</sup>.

In adult rats the total circulating haemoglobin and plasma proteins were depressed by about 50 per cent by maintaining them on protein-free diets for a period of 8 weeks. They were then fed known quantities of vegetable protein mixtures. A group repleted with skim milk served as a control. Total circulating haemoglobin and plasma protein were studied at different intervals during the period of repletion. The results of these experiments are depicted in Figures 2 and 3.

The results show that vegetable protein mixtures are effective in the regeneration of haemoglobin and plasma proteins although the time taken in the case of vegetable protein diets to attain the optimum levels was a little longer than with skim milk.

### Vitamin B<sub>12</sub> and vegetable proteins

Animal protein foodstuffs contain vitamin B<sub>12</sub> which, on the other hand, is absent or is present only in negligible quantities in vegetable protein foodstuffs. The possibility



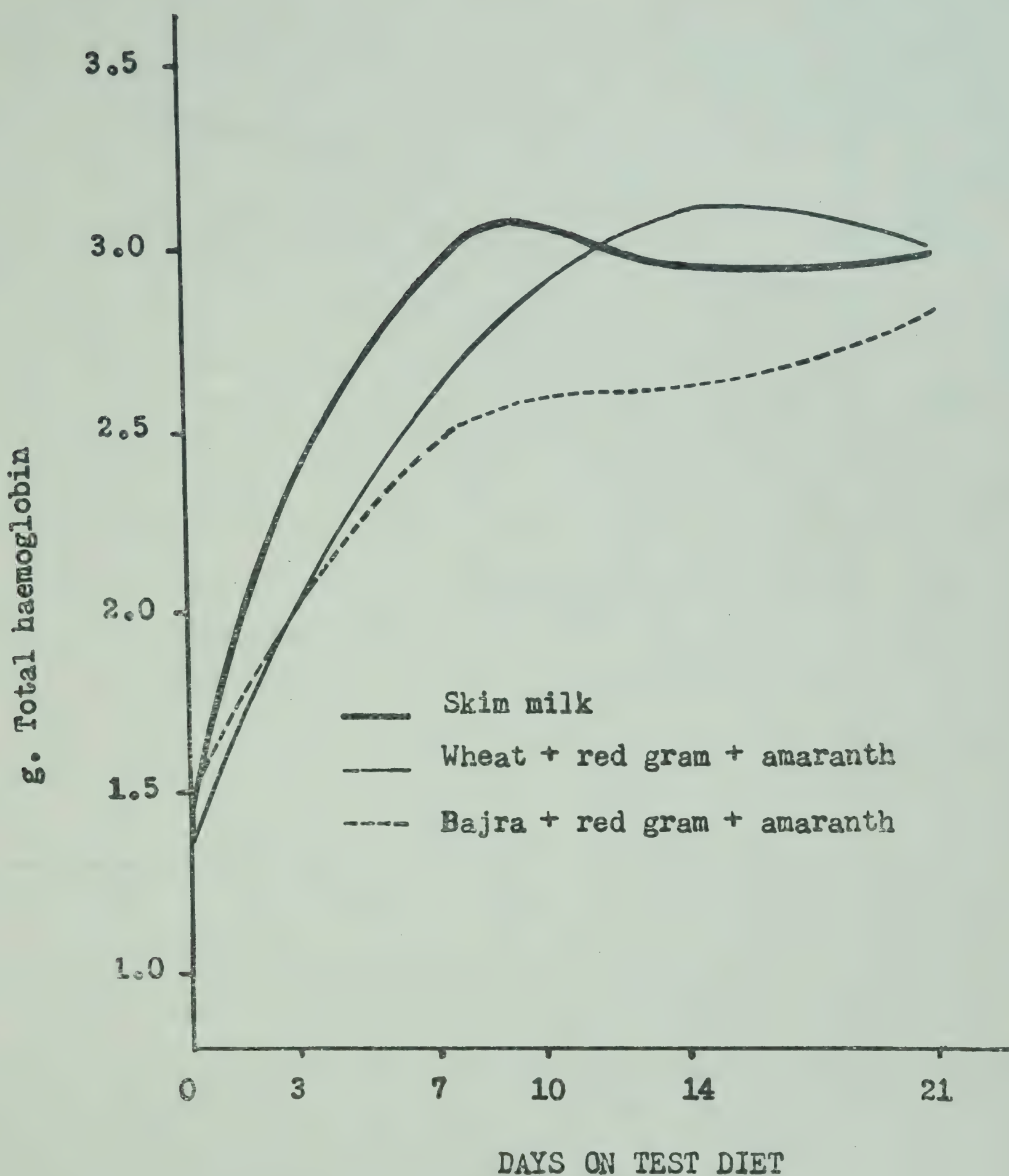


FIGURE 2. Regeneration of haemoglobin during protein repletion.

of vitamin B<sub>12</sub> deficiency in animals maintained on vegetable proteins had to be considered. We, therefore, utilised the opportunity to determine the vitamin B<sub>12</sub> status of the animals maintained on vegetable protein diets.<sup>12</sup>

Vitamin B<sub>12</sub> was estimated microbiologically in diets, faeces, liver and serum using *L. leichmanii* at the end of the feeding periods and the results are summarised in Table III.

The vitamin B<sub>12</sub> content of the experimental diets was negligible as compared to the stock diet, whole egg and skim milk diets. The faeces showed vitamin B<sub>12</sub> activity after 8 weeks in both the animal and vegetable protein groups. The vitamin B<sub>12</sub> activity in



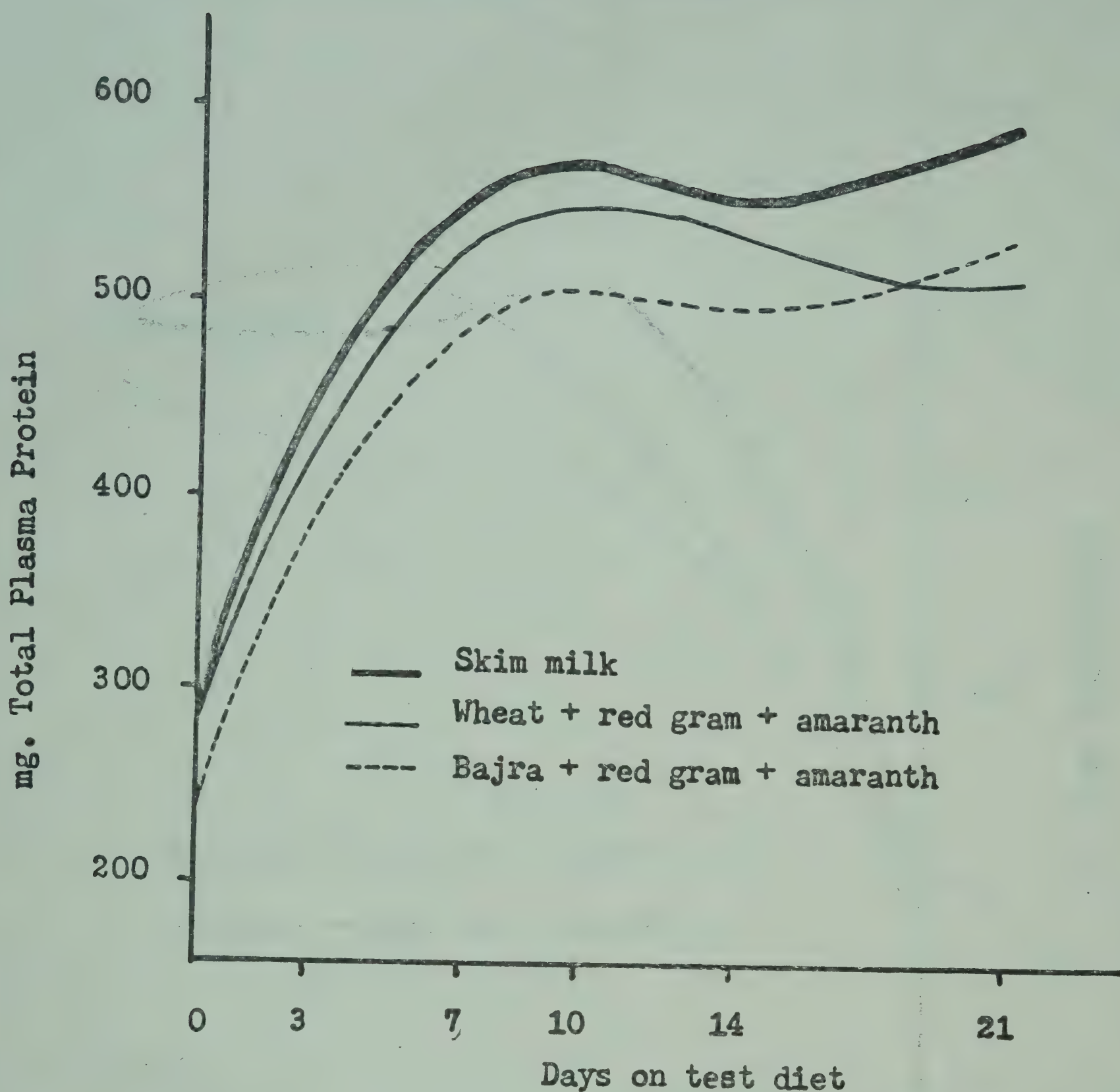


FIGURE 3. Regeneration of total plasma protein during protein repletion.

the whole liver was increased after feeding the experimental diets over that at the beginning. Serum also showed vitamin  $B_{12}$  activity in all groups and these values did not show a fall even after six months of feeding vegetable protein diets. Barnes and Fiala<sup>13</sup> reported that rats obtained their vitamin  $B_{12}$  requirement through coprophagy. They were not able to produce vitamin  $B_{12}$  deficiency unless coprophagy was prevented. Satyanarayana *et al.*<sup>14</sup> have studied this aspect further and their results indicate that there is a reduction in  $B_{12}$  activity in serum and total liver when coprophagy is prevented. While translating the results on experimental animals to human nutrition one will have to bear in mind the possibility of vitamin  $B_{12}$  deficiency occurring in humans subsisting for long periods entirely on vegetable proteins.

### Vegetable proteins and fatty livers

It is now realised that quantity as well as quality of proteins plays an important role in the prevention of dietary liver damage. The work of Shils and Stewart<sup>15</sup> who observed fatty



TABLE III  
Vitamin B<sub>12</sub> activity of livers and serum

Diet	Number of animals	Body weight g	Liver weight g	True B <sub>12</sub> Activity Whole Liver:		Serum $\mu$ mg/cc.
				$\mu$ g.	S.E.	
Weanlings ...	7	45.0	1.88	0.402	0.0126	...
Stock diet ...	8	186.1	7.29	0.925	0.0431	1.60
Whole egg ...	8	232.6	9.41	2.624	0.2295	...
Skim milk ...	8	213.4	7.48	1.112	0.0498	2.01
Bajra + red gram + amaranth	8	201.0	7.99	0.881	0.0674	...
Rice + red gram + amaranth	8	190.0	7.41	1.080	0.0672	1.77
Wheat + red gram + amaranth	7	197.7	8.24	1.271	0.0735	1.87
<b>AFTER 24 WEEKS</b>						
Wheat + red gram + amaranth	7	348.4	12.71	1.908	0.2742	1.98
Stock diet ...	8	329.2	12.65	1.668	0.1396	1.65

livers in animals fed on vegetable foods and disappearance of this lesion by supplementing the limiting amino acids led us to undertake the investigation on incidence of fatty livers on vegetable protein diets used by us<sup>16</sup>.

Three groups of young rats were kept on the following diets for 4 to 8 weeks: (1) protein from cereal alone at 10 per cent level, (2) 6 parts of protein from cereal, 3 parts from a pulse and 1 part from amaranth leaf, and (3) skim milk at 10 per cent protein level. All the diets had adequate choline and 5 per cent fat. The livers of the animals fed single cereal diets showed mild to moderate periportal fatty change as has been observed by Harper<sup>16</sup> in single amino acid deficiency. The animals receiving mixtures of vegetable proteins, however, did not show any fatty infiltration in most cases. This probably is due to the correction of amino acid deficiency and imbalance by mutual supplementation.

These results show the importance of histological study of the liver in the nutritive evaluation of proteins.

## Discussion

All the results presented above clearly show that a mixed vegetable protein diet in the proportions used does have a good nutritive value as judged by its efficiency to promote growth of young animals. The Food and Agricultural Organisation in their recommendations on protein requirements have given an ideal pattern of essential amino acids which, according to them, is better than that present in egg<sup>18</sup>. This pattern is based on the work of essential amino acid requirements for maintenance and growth carried out by different workers on human subjects.

A comparison of the FAO pattern with the pattern of essential amino acids in two cereal proteins and two typical vegetable protein mixtures is shown in Table IV.

As can be seen, there is not much difference between the essential amino acid composition of bajra and rice proteins alone, although the former has a lower PER than the latter.



TABLE IV

*Essential amino acid content of some vegetable proteins and their blends  
as compared with FAO provisional pattern (g per 100 g protein)*

		Bajra protein	Mixture: Bajra protein 6 + red gram protein 3 + amaranth protein 1	Rice protein	Mixture: Rice protein 6 + red gram protein 3 + amaranth protein 1	FAO pattern
Isoleucine	...	5.9	5.7	6.0	5.8	4.2
Leucine	...	9.5	8.6	8.0	7.7	4.8
Lysine	...	3.8	4.9	3.9	4.9	4.2
Methionine + cystine	...	3.7	2.9	4.0	3.1	4.2
Phenylalanine	...	4.3	5.7	4.6	5.9	2.8
Threonine	...	3.8	3.7	3.3	3.6	2.8
Tryptophan	...	1.9	1.3	1.2	0.9	1.4
Valine	...	6.3	6.0	6.1	5.9	4.2

The biological value of both these cereals, however, is the same (Table I). Rice and rice-pulse-amaranth mixture does not have a different PER, although there is some improvement in lysine content. With this improvement, however, bajra-pulse-amaranth mixture has a higher PER, than bajra alone.

These facts are really puzzling and one wonders whether the close approach to the FAO pattern will necessarily indicate a high nutritive value. Possibly, the essential amino acid content of the protein is not the only determinant to arrive at the nutritive value either for maintenance or for growth.

We did find a difference in the regression equation between biological value and the chemical score from that of Block and Mitchell in case of vegetable proteins as depicted in Figure 4.

One finds from this figure that the vegetable proteins are scattered far apart from the regression line of Mitchell. The animal proteins, on the other hand, are quite close to the line. We have plotted another line from our data on vegetable proteins but it is different from that of Mitchell. Here again, the variation is wide. A vegetable protein having the same score as that for animal protein shows a higher biological value than the latter. This again goes to prove that we are dealing, perhaps, with an entirely different situation in the case of vegetable proteins.

Longnecker and Hause<sup>19</sup> have estimated plasma essential amino acids over a period of five hours after a meal. They calculated from these figures the pattern of essential amino acids actually available to the animal. The deficit of limiting amino acid as observed by them is different from that in the protein. Perhaps the pattern of essential amino acid available *in vivo* could be different.

Another question which vegetable proteins pose is that of imbalance of amino acids. Although workers on vegetable proteins are well aware of the work of Elvehjem and his school, they have not undertaken investigations on this aspect presumably because of the difficulty of procuring pure amino acids in quantity. This aspect, however, deserves to be looked into.



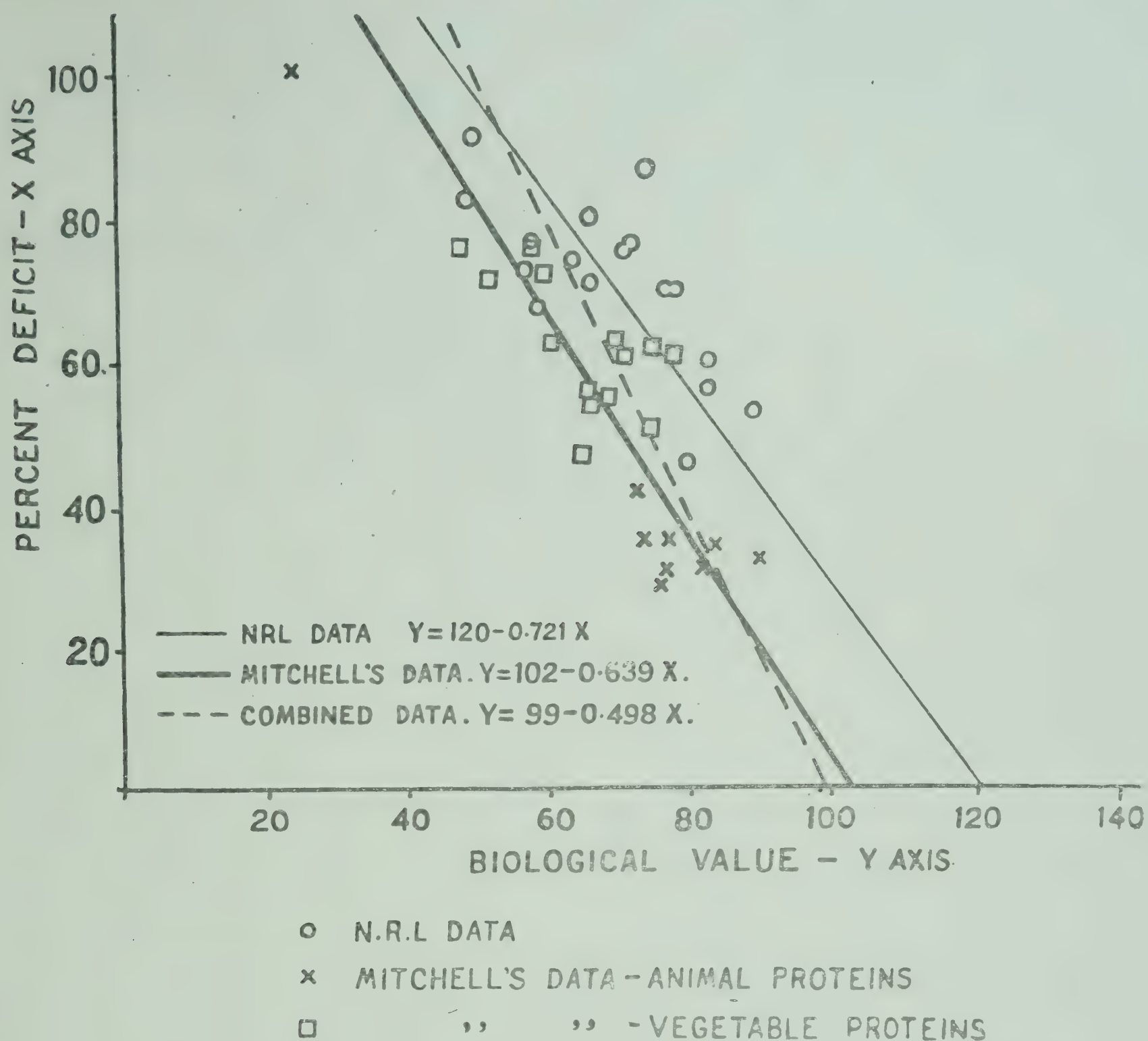


FIGURE 4. Correlation between percentage deficit in the limiting essential amino acid and biological value

One cannot overlook the results of PER in relation to net protein utilisation as advocated by Bender. The argument that the body puts on more fat in some cases and gives a false PER which could be low from the point of view of net protein utilised is definitely valid and at the Nutrition Research Laboratories, we hope to take up the examination of this aspect along with the others stated above in the near future to enlarge our understanding of nutritive value of vegetable proteins.

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# BIOLOGICAL VALUE AND ESSENTIAL AMINO ACID COMPOSITION OF THE PROTEINS OF SOME PULSES

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As pulses constitute important ingredients of our diet, it was of interest to determine the biological value of the proteins of pulses which are commonly used. The biological value, however, depends on the essential amino acid composition of the protein. The essential amino acid composition of seven varieties of pulses and rice, therefore, were contents of determined by the microbiological assay procedure.

## Materials and Methods

The biological value was determined by the rat-growth method of Osborne *et al.*<sup>1</sup> The biological values were calculated from the total protein intake and the total gain in weight in 8 weeks.

The ten essential amino acids were determined by the microbiological assay procedure described by Barton-Wright<sup>3</sup>. Leucine, isoleucine, valine and tryptophane were estimated with *Lactobacillus arabinosus*; methionine, phenylalanine, lysine, and histidine with *Luconostoc mesenteroides* and threonine and arginine with *Streptococcus faecalis*. Protein samples were determined by the Kjeldahl method.

## Results and Discussion

The results are given in Tables I-III. Among the pulses tested the protein efficiency ratio (PER) was highest in the case of *Phaseolus radiatus* and least in the case of *Lens esculenta*. The essential amino acids are present in varying quantities in different pulses. Among them, *Lathyrus sativus* was found to contain the largest amount of most of the essential amino acids. If the total amount of the ten essential amino acids are taken into account, then the pulses can be placed in the following order of superiority with respect to their amino acids content: *Phaseolus mungo*, *Phaseolus radiatus*, *Pisum sativum*, *Cajanus indicus*, *Lens esculenta* and *Cicer arietinum*. Protein contents of pulses was highest in *Lathyrus sativus* and lowest in *Phaseolus radiatus*. If the essential amino acid contents of samples are expressed in relation to their protein

TABLE I  
*The biological value of proteins of pulses*

Pulse	PER
Cicer arietinum ... ..	1.72
Phaseolus mungo ... ..	1.51
Lens esculenta ... ..	1.16
Phaseolus radiatus ... ..	1.87
Pisum sativum ... ..	1.53
Casein ... ..	1.95



TABLE II  
Essential amino acid contents of pulses and rice  
(g per 100 g sample)

	Phaseolus radiatus	Lens esculenta	Phaseolus mungo	Cicer arietinum	Pisum sativus	Lathyrus sativus	Cajanus indicus	Parboiled rice
Leucine ...	2.10	1.96	2.24	1.28	2.00	2.33	1.82	0.41
Iso-leucine ...	1.67	1.50	1.60	1.07	1.50	2.17	1.38	0.22
Valine ...	1.45	1.31	1.52	0.85	1.33	1.75	1.19	0.33
Phenylalanine ...	0.97	0.86	1.17	0.71	0.96	1.04	1.73	0.21
Methionine ...	0.22	0.12	0.33	0.18	0.17	0.14	0.19	0.09
Histidine ...	0.42	0.39	0.44	0.26	0.45	0.52	0.47	0.09
Lysine ...	1.57	1.70	1.60	1.02	1.86	1.86	1.46	0.18
Arginine ...	1.74	1.96	1.64	1.45	2.81	3.13	1.60	0.43
Threonine ...	0.69	0.91	0.74	0.60	0.89	1.18	0.85	0.20
Tryptophane ...	0.09	0.08	0.09	0.08	0.05	0.07	0.03	0.04
Protein (%) ...	17	23	22	19	21	25	21	7

TABLE III  
Essential amino acid contents of pulses and rice  
(g per 100 g protein of the sample)

	Phaseolus radiatus	Lens esculenta	Phaseolus mungo	Cicer arietinum	Pisum sativus	Lathyrus sativus	Cajanus indicus	Parboiled rice
Leucine ...	12.35	8.52	10.18	6.74	9.52	9.32	8.76	5.91
Iso-leucine ...	9.85	6.52	7.27	5.66	7.14	8.68	6.57	3.08
Valine ...	8.53	5.70	6.93	4.47	6.53	7.00	5.67	4.71
Phenylalanine ...	5.73	3.74	5.31	3.74	4.56	4.16	8.24	3.00
Methionine ...	1.28	0.53	1.50	0.94	0.80	0.56	0.89	1.32
Histidine ...	2.47	1.70	2.00	1.38	2.14	2.08	2.24	1.22
Lysine ...	9.24	7.39	7.27	5.37	8.86	7.44	6.95	2.51
Arginine ...	10.24	8.52	7.45	7.63	13.38	12.52	7.62	6.14
Threonine ...	4.06	3.95	3.36	3.16	4.24	4.72	4.05	2.86
Tryptophane ...	0.54	0.34	0.40	0.45	0.26	0.27	0.17	0.61

content (Table III) then the pulses may be arranged in the following order with respect to their essential amino acid composition: *Phaseolus radiatus* *Pisum sativus*, *Lathyrus sativus*, *Phaseolus mungo*, *Cajanus indicus*, *Lens esculenta* and *Cicer arietinum*. *Lathyrus sativus* is rich in protein and essential amino acids. The cultivation of this pulse should, therefore, not be banned unless it is proved conclusively that it is the cause of lathyrism. Rice is very rich in tryptophane, methionine, phenylalanine and histidine.

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# HIGH AND LOW PROTEIN BENGAL GRAM: NITROGEN DISTRIBUTION AND NUTRITIONAL ASSESSMENT

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It has been reported that strain and/or environmental conditions influence the total crude protein content of the same variety of pulse to a significant extent<sup>1</sup>. This total pulse protein is, however, composed of different protein fractions. But it is not known whether any variation occurs in the proportions of these different constituent proteins in the above high and low protein strains. Only limited work has so far been done on the isolation and fractionation of all the constituent proteins in pulses. A few workers have studied only single isolated fractions<sup>2,3</sup> of pulse proteins.

Pulses usually contain significant amounts of non-protein nitrogen (NPN)<sup>4,5</sup>. The extent to which such nitrogen content influences the overall nutritive value of both low- and high-protein pulses is not known.

The ultimate nutritive value of a food protein depends, besides its protein fractions, on its overall amino acid pattern. Results of amino acid analyses of a few varieties of pulses have been reported from time to time<sup>6-13</sup>. These, however, are often found to vary in the same variety of pulse. Whether such variations are due to variation in protein fractions in different samples is not clearly understood.

In view of the above considerations, it was considered desirable to study the variations if any in protein fractions, NPN and amino acid composition in high and low protein Bengal gram, induced under the influence of strain and / or environment, which may influence their overall nutritive value.

## Experimental procedure

Pure bred Bengal gram samples with high- and low-protein contents, as collected from different State Agricultural Farms and Research Institutes, were dehusked and finely powdered to pass through 60 mesh seive. The samples tested along with their strains and environmental conditions under which they were raised are shown in Table I.

*Crude protein percentage:* Crude protein ( $N \times 6.25$ ) percentage of each sample was estimated by Kjeldahl method.

*Fractionation of protein:* Fractionation of defatted pulse proteins was conducted by the customary solubility method<sup>14</sup>. The optimum conditions for extraction by different solvents were worked out. The full scheme of fractionation is given in Chart 1. Defatting of the samples was done according to the method of A.O.A.C. (1950).

*Paper electrophoretic study* was done with isolated purified albumin and globulin fractions in a Tiselius type of electrophoretic apparatus\*. The method of purification

\* Fabricated in this Institute by Mr B. K. Bardhan.

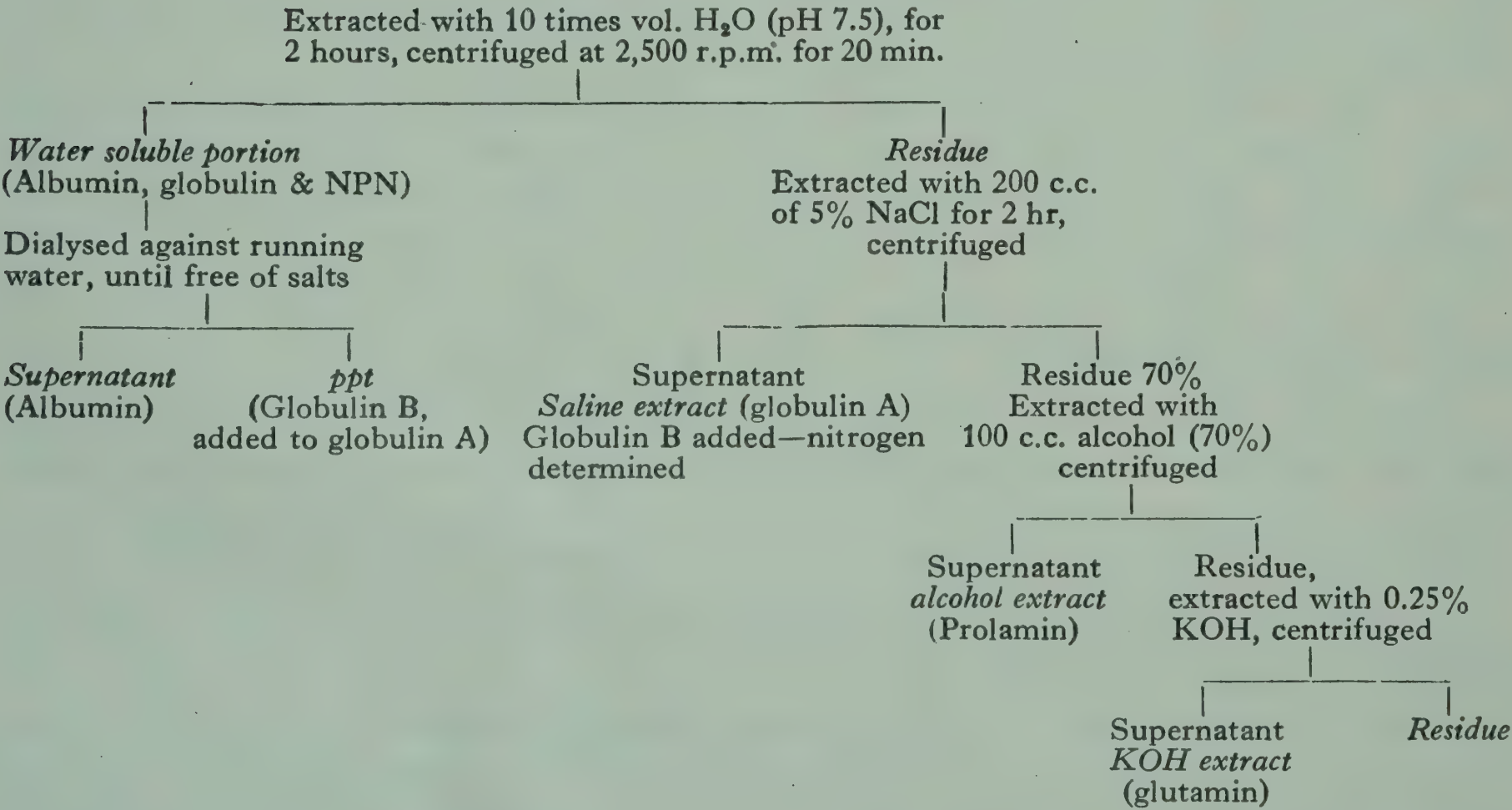


TABLE I

The strains of the samples used in the investigation and the environments where they were raised

Sample No.	Crude protein (on dry basis) %	Strain	Locality	Environmental conditions	
				Annual rainfall (cm)	Type of soil
LOW-PROTEIN					
1	17.9	Pink 2	Ujjain	80-90	Black cotton
2	18.3	152-4-14-17-7	Kopargaon	...	...
3	19.7	1.P.1. 707	Indore	90-100	Black cotton
4	19.9	Adt. V.	Nagpur	100-120	Black cotton
HIGH-PROTEIN					
5	26.3	S.98	Berhampur	120-140	Alluvial
6	26.8	Chafa	Junagadh	60-70	Black cotton
7	27.9	57.86	Parbani	...	...
8	27.9	S.10	Berhampur	120-140	Alluvial

CHART 1  
Fractionation Chart  
Defatted pulse sample (20g)



of isolated albumin and globulin protein is shown in Chart 2. Electrophoresis was conducted with a constant current for 16 hours at room temperature under a voltage gradient of 220 V. Filter paper (Whatman No. 1, 36 cm×15 cm) previously washed with 0.1 N HCl and dried in air was used for this purpose and buffers of the following composition (as shown in Table II) were employed. 0.05 per cent bromophenol blue



CHART 2

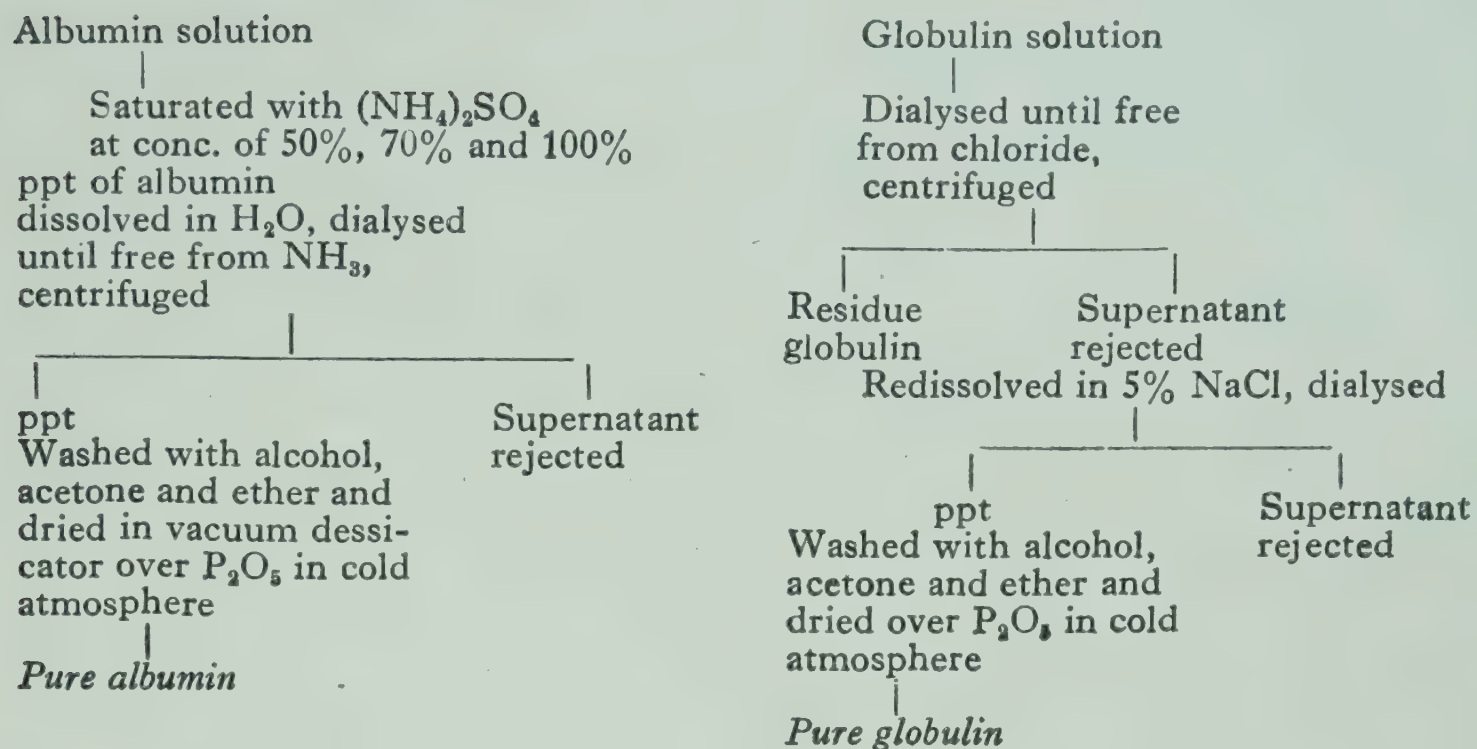
*Isolation of purified albumin and globulin*

TABLE II

*Buffers used for electrophoresis*

No.	Composition	pH
1	0.046M $\text{NaH}_2\text{PO}_4$ 0.020M $\text{Na}_2\text{HPO}_4$	6.4
2	0.0015M $\text{NaH}_2\text{PO}_4$ 0.0316M $\text{Na}_2\text{HPO}_4$	8.0
3	0.05M Sodium Veronal 0.01M Veronal	8.6
4	0.2M $\text{NH}_3$ 0.1N $\text{NaCl}$	9.26

saturated with  $\text{HgCl}_2$  was used for staining purpose. Final rinsing and washing was conducted according to the method employed by Zencks *et al.*<sup>15</sup>.

**NPN:** The NPN. was determined according to the method adopted by Kulkarni *et al.*<sup>5</sup> using 2.5 per cent trichloroacetic acid as protein precipitant. The  $\alpha$ -amino nitrogen in NPN fraction was estimated by Sorensen's formal titration method.

**Amino acid determination:** For the purpose of amino acid determination 1 g of pulse powder was hydrolysed with 15 ml of 5.7 N  $\text{HCl}$  at  $105^\circ\text{C}$  for 24 hours according to Holmes<sup>16</sup>. Excess of  $\text{HCl}$  was removed by repeated evaporation under suction. After decolorising by Darco 60, the volume of hydrolysate was made up to 20 ml with 10 per cent isopropyl alcohol. Estimation of the amino acids was made by two dimensional paper chromatographic method according to Consden *et al.*<sup>17</sup> using phenol-water and butanol-acetic acid-water as solvents. Tryptophan was estimated chemically according to the method of Graham *et al.*<sup>18</sup> using *p*-dimethylamino benzaldehyde.



## Results and Discussion

The results of fractionation are shown in Table III.

TABLE III  
*Fractionation of the proteins of high and low protein pulse samples*

Extractant	Total nitrogen dispersed in low-protein sample %		Average	Total nitrogen dispersed in high-protein sample %		Average
	1	2		7	8	
Water (albumin + globulin + NPN) ... ..	34.8	35.0	34.9	33.5	33.7	33.6
Albumin ... ..	4.2	4.0	4.1	2.7	3.0	2.8
Globulin B ... ..	10.1	10.3	10.2	9.3	10.0	9.7
NPN ... ..	20.5	20.7	20.6	21.5	20.7	21.1
5% NaCl (globulin A) ... ..	56.0	54.2	55.1	50.8	52.2	51.5
Total globulin ... ..	67.1	64.5	65.8	60.1	62.2	61.2
70% Alcohol (prolamin) ... ..	0.5	0.4	0.5	0.3	0.2	0.3
0.25% KOH (glutamin) ... ..	3.2	3.7	3.4	8.2	6.9	7.5
Residue ... ..	2.0	2.1	2.1	2.7	3.2	2.9

All values are the average of three determinations.

The results indicate that in both high- and low-protein Bengal gram, the successive extraction with water and 5 per cent NaCl solubilizes 85-90 per cent nitrogen. This amount to nitrogen is contributed by albumin, globulin and NPN of the protein, globulin forms the major fraction constituting more than 60 per cent of total nitrogen. Both albumin and globulin fractions were found to decrease very slightly in high protein pulses, whereas glutamin fraction was found to increase slightly in these samples. Prolamin was found to be present in very small amount. In the purification of isolated albumin 75-80 per cent of total albumin could be recovered by precipitation with full saturation of  $(\text{NH}_4)_2\text{SO}_4$ . This purified albumin fraction in both high- and low-protein samples was found to be homogeneous by electrophoresis with phosphate buffer at pH 8. The globulin fraction isolated and purified by dialysis against running water constituted only 20 per cent of the total globulin in pulses. This purified globulin was found to contain only one component by electrophoresis with veronal buffer at pH 8.6. This was in accordance with Nath *et al.*,<sup>19</sup> who found only one component in pure sesame seed globulin, isolated by dialysis.

The results of NPN analysis is shown in Table IV. The results of NPN analysis tend to indicate that NPN constitutes a considerable portion (more than 20 per cent) of total N in both high- and low-protein Bengal gram pulses. With the increase of protein



TABLE IV  
Summary of NPN analysis in high and low protein Bengal gram

Sample No.	Nitrogen/100 g of defatted sample (mg)		NPN as % of TN	Amino N mg/100 g of defatted sample	Complexity ratio = NPN $\alpha$ -amino N
	Total N TN	NPN			
	(mg %)				
1	3,456.52	739.70	21.4	250.75	2.95
2	3,529.10	779.93	22.1	259.11	3.01
3	3,640.64	859.19	23.6	299.37	2.87
5	4,257.80	1,064.45	25.0	351.30	3.03
6	4,232.22	1,070.33	25.28	319.46	3.35
7	4,689.00	1,134.73	24.2	387.62	2.93

content, both the NPN fraction and  $\alpha$ -amino nitrogen tended to increase very slightly keeping the complexity ratio almost identical in all cases. This suggests higher assimilation of nitrogen from high protein Bengal gram. Previous work<sup>20</sup> indicates greater digestibility for high protein Bengal gram. From the preliminary study on the amino acid composition the results show that all the amino acids studied were present in both high- and low-protein pulses. The amount of each of these amino acids tended to increase with an increased amount of protein in Bengal gram pulse. However, the increase of all the amino acids did not necessarily correlate directly with increase in protein content. These amino acid patterns are being studied microbiologically with a view to confirming the present findings. The slightly lower biological values of protein in high protein pulses as determined by the balance sheet method<sup>20</sup>, however, suggests the possibility of a little altered amino acid pattern.

The above findings suggest that the increased protein in high protein pulses as induced by strain and/or environments does not differ appreciably from that of low protein pulses both in the nitrogenous fractions and amino acid composition. The slight differences are not likely to influence the overall dietary value especially when in mixed diets, and as such from economic and agronomical points of view, it seems that growing of pulses with high protein content would be useful.

Further work regarding the influence of fertilizers and other agronomical variations both on the increase of protein content and improvement of its quality is contemplated.

### Summary

High and low protein Bengal gram grown with different strains and under different environmental conditions were fractionated by the customary solubility method. In both high and low protein samples, the successive extraction with water and sodium



chloride (5 per cent) solubilized 85-90 per cent N. Globulin formed the major fraction (more than 60 per cent) and NPN was found to constitute more than 20 per cent of total N. In high protein samples albumin as well as globulin fraction tended to decrease slightly whereas glutin and NPN tended to increase to a minute extent. No appreciable difference was found in the amino acid composition between the high- and low-protein samples as determined by paper chromatography. The nutritional significance of the high protein content in pulses has been discussed.

### Acknowledgment

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# NUTRITIONAL EVALUATION OF VEGETABLE PROTEIN ISOLATES AND THEIR FORMULATIONS

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The processing of vegetable materials for the isolation of edible proteins is a development of recent origin. In view of their high protein content and bland flavour, isolated proteins are specially suited for the preparation of protein-rich foods for infants and children. Among the various sources for the production of protein isolates, oilseeds and oilseed meals occupy the prime place because of their relative abundance and ease of processing. Edible soyabean protein is produced in the U.S.A. on a commercial scale and is finding increasing application in the form of both familiar and new types of foods<sup>1</sup>. Pilot plants for the economic production of groundnut protein are at present in operation in India<sup>2</sup> and elsewhere<sup>3</sup> and these efforts may lead to the large scale production of the protein isolate for various food uses.

The nutritive value of groundnut as a source of dietary protein has been investigated by many workers. Majority of the studies have been made with groundnut meal or the defatted oil cake rather than with isolated proteins<sup>4,5,6</sup>. The low biological value of groundnut protein is due to the poor pattern of essential amino acids, the limiting amino acid being methionine; secondary deficiencies are lysine and possibly threonine and isoleucine<sup>7,8</sup>. Amino acid analyses show that there is little difference between the composition of the isolate and that of the meal<sup>9</sup>.

Grau<sup>8</sup> demonstrated the beneficial effects of supplementing groundnut meal with methionine on growing chicks and Cama and Morton<sup>10</sup> showed that the addition of methionine to the meal improved the protein efficiency ratio in albino rats. Marais and Smuts<sup>11</sup>, on the other hand, failed to obtain any significant improvement in the growth rate of rats with methionine supplemented meals. Altschul<sup>12</sup> found that isolated groundnut protein had about the same nutritional value as groundnut meal when fed as a supplement in chick rations. Murphy and Dunn<sup>13</sup> found that at 19 per cent protein level in the diet the isolate had to be supplemented with lysine and methionine for satisfactory reproduction and lactation in the case of mice. It was, therefore, of interest to assess the nutritive value of the protein isolate from the kernel or the cake and study the effect of supplementation with amino acids and other proteins.

## Experimental materials and methods

Groundnut protein isolate for the present studies was prepared from edible expeller cake as described in an earlier paper<sup>14</sup>. Protein isolate from commercial quality cake and that obtained from the kernel by the Integrated Process<sup>15</sup> were used for comparison in one of the studies. Soya protein isolate was prepared from American Multipurpose Food which is based mainly on toasted soya grits. Bengal gram flour and hexane-extracted white sesame cake were used for isolating the respective proteins. Commercial lactic casein was used for comparison and also in the different formulations. *DL*-methionine (Merck) and *L*-lysine monohydrochloride (B.D.H.) were used along with the isolates in supplementation studies.



The various isolates were analysed for their protein ( $N \times 6.25$ ) and fat content and incorporated either singly or as blends in adequate diets at 10 per cent protein level.

The digestibility coefficient and biological value of the proteins were determined on growing male adult rats weighing about 150 g each. The growth promoting value and the protein efficiency ratio of the diets were determined on comparable groups of weanling rats over a period of eight weeks. Nitrogen retention studies were also carried out during the first three weeks of the experimental period in one set of growth studies.

## Results and Discussion

The digestibility coefficient and biological value of the protein isolates from the kernel, edible expeller cake and the commercial cake as compared to casein and the whole cake proteins are given in Table I. The digestibility coefficient of the isolates range from

TABLE I  
*Digestibility coefficient and biological value of groundnut  
protein from kernel and expeller cake  
(10% protein level in the diet of experimental rats)*

Source of protein	Digestibility coefficient	Biological value
Groundnut protein isolate from kernel (Integrated process)	97.9	52.1
Protein isolate from commercial cake ...	96.8	53.8
Protein isolate from edible cake ...	97.2	51.9
Edible cake protein isolate + 6% <i>DL</i> -methionine ...	98.2	59.7
Edible cake protein isolate + casein (1:1) ...	96.6	62.3
Groundnut cake, whole meal (control) ...	90.1	56.0
Casein (control) ...	96.5	73.7

96.8 to 97.9 as compared to 90.1 for the whole cake proteins. The higher digestibility coefficient of the isolate is due to the elimination of fibre and other insoluble carbohydrate fractions from the cake during the process of protein isolation. The biological value of the isolates range from 51.9 to 53.8, the differences being not significant. As compared to a biological value of 56.0 for the whole edible cake proteins, the isolate from it gave a value of 51.9, indicating a slight lowering in protein utilization on isolation. This may presumably arise from the loss of water-soluble non-protein nitrogenous fractions in the whey during the isolation of protein. Supplementation of groundnut protein isolate with *DL*-methionine at 6 per cent level enhanced the biological value significantly and was comparable to a 1:1 blend of groundnut protein and casein fed at similar levels.

The growth promoting value of groundnut protein isolate and a 1:1 mixture of groundnut protein and casein with and without supplementation with lysine and methionine are given in Table II. The isolate (Group B) has given a protein efficiency ratio of 1.42 as compared to 2.32 for casein (Group A) over a period of eight weeks. A mixture of casein and groundnut protein in equal proportions (Group C) has given a value of 1.91, which is close to the arithmetic mean of the protein efficiency ratio of the two components. This suggests that there is no true supplementary effect between groundnut protein and casein under the conditions of the experiment. Similar conclusions can also be drawn from the data on the biological value<sup>16</sup>. The absence of mutual supplementation may be



TABLE II

*Protein efficiency ratio of groundnut protein with supplements and nitrogen retention values*  
(8 weeks duration)

(12 weanling rats per group—10 per cent proteins, level in diets)

Source of protein	Average intake		Average gain in weight (g)	Average PER	N Retention %
	Diet	Protein			
A. Casein ...	405.6	40.8	95.3	2.32	...
B. Groundnut protein ...	349.0	36.8	52.3	1.42	33.8
C. Groundnut protein + casein (1:1) ...	384.1	39.6	75.7	1.90	...
D. Groundnut protein + 2.1% methionine + 1.7% lysine ...	379.7	40.2	79.5	1.98	48.0
E. Groundnut protein + casein (1:1) + 3.9% methionine + 4.25% lysine ...	466.3	48.8	126.3	2.53	66.0

Equivalent amount of *L*-lysine, HCL and *DL*-methionine were used.

due to the fact that this mixture at 10 per cent level in the diet is still inadequate to meet the requirements of all essential amino acids for the optimal growth of rat. In Group D the protein isolate has been supplemented so that the proportion of tryptophan: methionine: lysine is optimum (1:3:5), as suggested by Rose<sup>17</sup>, for the normal growth of rats. This has brought about a significant improvement in the protein efficiency ratio as well as nitrogen retention and the results are comparable with Group C receiving an equal mixture of groundnut protein and casein. The last group (Group E) receiving a mixture of casein and groundnut protein with larger supplements of lysine and methionine has given a PER of 2.53; also the nitrogen retention is as high as 66 per cent.

The average gains in weight of the experimental animals have been plotted against the nitrogen consumed in the case of the different diets (Fig. 1). It is seen that the amounts of nitrogen required to promote an initial gain in weight of 50g in the case of casein (Group A), groundnut protein (Group B) and a mixture of groundnut protein and casein with maximal amino acid supplements (Group E) are 2.75g, 5.8g and 2.4g respectively, whilst, on a mixture of groundnut protein and casein (Group C), or on groundnut protein alone with optimal supplements of both lysine and methionine (Group D), the requirement of nitrogen is 3.9 g. The growth promoting values of blends of different vegetable protein isolates, viz., groundnut, soya, Bengal gram (*Cicer arietinum*) and sesame (*Sesamum indicum*) with or without casein and amino acids, as evaluated on weanling albino rats are given in Table III. Skim milk powder has also been included along with casein for comparison in this study.

Proteins from groundnut, soya, and blends of groundnut-soya (1:1) and groundnut, Bengal gram and sesame (5:3:2) gave PERs of 1.53, 1.56, 1.66 and 1.61 respectively. Blends of groundnut, soya and casein (3:3:4) or soya and casein (1:1) gave a value of 1.8 for PER. A mixture of groundnut and soya proteins require to be supplemented with 2 per cent *L*-lysine and 3 per cent *DL*-methionine to raise the PER from 1.7 to 1.9, while a blend of 60 per cent vegetable proteins and 40 per cent casein required lower levels of amino acid supplements to make the growth promoting value comparable to skim milk powder. Soya



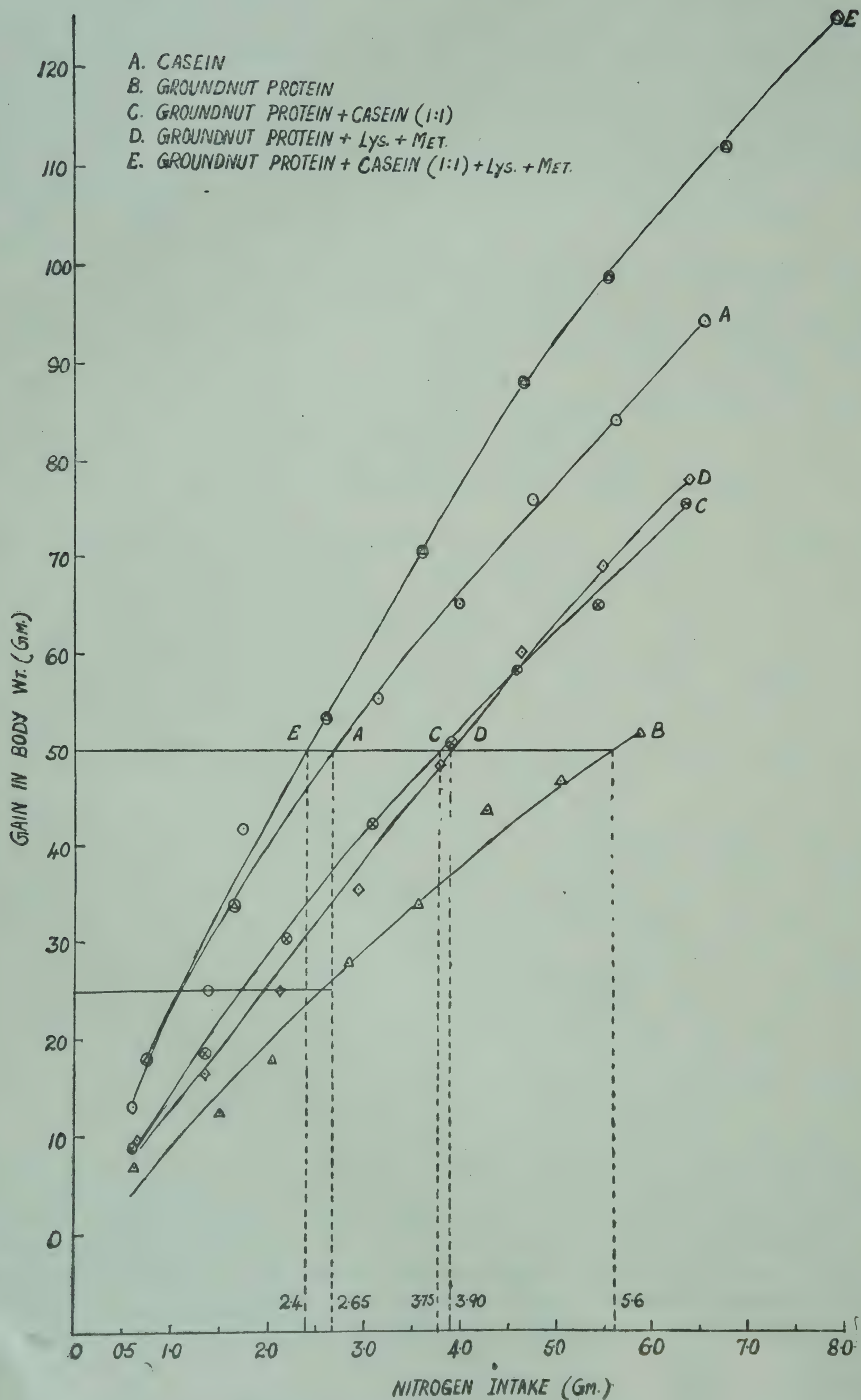


FIG. 1. Nitrogen intake vs. gain in weight.



TABLE III

*Comparative protein efficiency ratios of skim milk powder, casein, vegetable protein isolates and their blends with and without amino acid supplements*

(9 weanling rats per group—8 week duration—10 per cent protein level in diet)

Group	Source of protein	Average diet	Intake protein	Average gain in body wt (g)	Average PER
<b>SERIES I</b>					
A	Skim milk powder ...	630.7	66.5	150.0	2.13
B	Casein ...	561.0	60.9	123.0	1.98
C	Groundnut protein ...	488.8	54.0	83.2	1.53
D	Soya protein ...	427.3	45.9	72.0	1.56
E	Groundnut + Soya (1:1) ...	518.9	57.0	94.7	1.66
F	Groundnut + Soya (1:1) + 2.6% lysine + 3.0% methionine ...	544.6	61.6	120.6	1.91
G	Groundnut + Soya + Casein (3:3:4) ...	545.5	59.9	109.1	1.81
H	Groundnut + Soya + Casein (3:3:4) + 0.9% lysine + 2.4% methionine ...	574.8	65.0	137.6	2.07
I	Soya + Casein (1:1) ...	540.2	57.6	108.0	1.84
J	Soya + Casein + 1.8% methionine ...	571.7	62.4	139.5	2.15
K	Groundnut + Bengal gram + Sesame (5:3:2) ...	533.9	59.2	96.2	1.61
<b>SERIES II</b>					
A	Soya protein ...	521.4	54.7	92.3	1.67
B	Soya protein + 2.9% methionine ...	515.9	54.7	118.8	2.12

Equivalent amount of *L*-lysine, HCL and *DL*-methionine were used.

protein isolate or a mixture of soya protein and casein needs to be supplemented only with methionine to enhance the PER to that of skim milk powder.

## Summary

(1) Groundnut protein isolate and protein blends containing the isolates have been evaluated at 10 per cent level in adequate diets to assess their nutritive value. Studies on the supplementation of the isolates and their blends with casein and amino acids have also been carried out.

(2) Isolated proteins from groundnut kernel and expeller cakes are comparable in their biological value. The digestibility coefficient of the isolate is higher than that of the total proteins of the cake, but the biological value is slightly lowered on isolating the protein.

(3) Supplementation of groundnut protein with optimal quantities of lysine and methionine results in an increase in the protein efficiency ratio from 1.42 to 1.98. A mixture (1:1) of groundnut protein and casein has a PER of 1.91.

(4) The protein efficiency ratio of individual vegetable protein isolates from groundnut and soya, or a mixture (1:1) of these two or a blend containing groundnut, Bengal gram and sesame proteins range from 1.5—1.7. Blends containing 30 per cent groundnut protein, 30 per cent soya protein, and 40 per cent casein, or 50 per cent soya protein and 50 per cent casein possessed a PER of 1.8. These blends on further supplementation with lysine and methionine gave growth promoting values comparable to milk proteins.



(5) It appears that for an efficient utilization of groundnut protein or blends of vegetable proteins containing groundnut as a major component, supplementation with limiting amino acids or milk proteins is necessary.

### Acknowledgment

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### Discussion

- Q. Isn't the methionine supplementation rather high?
- A. The level of supplementation has been calculated to provide the optimal requirements of the rat for growth.
- Q. Are lysine and methionine available cheaply enough to make amino acid supplementation of vegetable protein diets practicable?
- A. Lysine is being produced commercially through microbiological and chemical synthesis. Methionine is being made at present by chemical synthesis only. Lysine is at present a little costly for large scale supplementation, but its price has fallen after the advent of microbiological procedures. It may be expected to fall further. Methionine is fairly cheap. Although there is no production of these amino acids in India at present, attempts at standardising microbiological and chemosynthetic processes for their production are being made in various laboratories in the country.



# THE NUTRITIVE VALUE OF PROTEIN BLENDS SIMILAR TO FAO REFERENCE PROTEIN PATTERN IN AMINO ACID COMPOSITION

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Naturally occurring proteins vary widely in their nutritive value, due primarily, to variations in their essential amino acid composition in relation to the requirements of animals or humans<sup>1, 2</sup>. Almost all vegetable proteins are partially deficient in one or more essential amino acids<sup>1, 3</sup>. Even though the proteins of legumes and oilseeds supplement those of cereals to a significant extent, they are, nevertheless, less effective than the proteins of milk and other animal foods<sup>4, 5</sup>. One way of improving the nutritive value of vegetable proteins as suggested by some workers is by supplementation of the proteins with the deficient essential amino acids<sup>7</sup>.

The need for utilising oilseed meals and pulses for making up the protein deficiency in the diets of children in several economically underdeveloped countries, where protein malnutrition is widely prevalent, has been engaging the attention of nutritionists and also of international organisations<sup>7, 10</sup>. Subrahmanyam *et al*<sup>9</sup>. reported that a blend of low fat groundnut flour (60 parts), Bengal gram (20 parts) and skim milk powder (20 parts) fortified with certain vitamins and calcium salts was very effective in the treatment of kwashiorkor. Scrimshaw *et al*.<sup>10</sup> recently reported that a protein food known as INCAP mixture 9B consisting of a mixture of corn, 29 parts, sorghum, 29 parts, cottonseed meal, 38 parts, dry torula yeast, 3 parts, calcium carbonate, 1 part and vitamin A, 10,000 I.U., was highly effective in the treatment of protein malnutrition in children. The recent report of the FAO Committee on protein requirements has emphasised the need for taking into consideration the amino acid content and nutritive value of the proteins present in the dietary for estimating human protein requirements<sup>11</sup>. Based on the available information on the amino acid requirements of children and adults, the FAO Committee recommended a 'reference protein pattern' which could serve as a standard of comparison in assessing the quality of dietary proteins. In the present investigation, the protein efficiency ratios of certain protein blends (with and without fortification with amino acids) having amino acid compositions similar to that of FAO 'reference protein pattern' have been studied. At the same time, it was considered to be of interest to determine the PER of protein blends which would prove useful in the treatment and prevention of protein malnutrition in children in different parts of the world.

## Experimental materials and methods

The samples of low fat groundnut flour and sesame flour used in the present investigation were prepared according to the method described by Subrahmanyam *et al*<sup>12</sup>.

Processed soya bean flour used in the present investigation was prepared according to Krishnamurthy *et al*<sup>13</sup>.



TABLE I  
*Amino acid composition of the different protein blends*  
 (Calculated per 16 g nitrogen)

Blend No.	Constituents				Arginine	Histidine	Lysine	Tryptophane	Phenylalanine	Methionine	Cystine	Threonine	Leucine	Isoleucine	Valine
B <sub>1</sub>	Soyabean + 55 parts	...	...	...						2.3 1.2					
	Sesame 45 parts	...	...	...	7.9	2.3	5.2	1.6	6.4	3.5		3.9	7.8	5.5	5.2
B <sub>2</sub>	Rice 88 parts	...	...	...						3.1 1.2					
	+ Skim milk powder 12 parts	...	...	...	7.9	2.0	5.8	1.3	5.0	4.3		3.9	9.2	6.6	6.5
B <sub>3</sub>	Skim milk powder 45 parts	...	...	...						3.2 1.2					
	+ Sesame 55 parts...	...	...	...	6.5	2.1	5.8	1.7	6.8	4.4		4.4	9.2	6.2	6.1
B <sub>4</sub>	Groundnut 45 parts	...	...	...						1.3 1.4		2.8	7.4	5.3	4.9
	+ Soyabean 55 parts	...	...	...	9.3	2.5	4.9	1.2	5.2						
B <sub>5</sub>	Groundnut 45 parts	...	...	...						2.7					
	+ Soyabean 55 parts	...	...	...	9.3	2.5	4.9	1.2	5.2	2.8 1.4		2.8	7.4	5.3	4.9
	+ Methionine 0.68 parts	...	...	...						4.2					
B <sub>6</sub>	INCAP 9B	...	...	...	10.8	2.8	3.7	1.3	6.1	1.9 2.1		3.6	8.3	4.6	5.5
										4.0					
	Skim milk powder	...	...	...	4.3	2.4	8.5	1.6	5.6	3.2 1.1		4.8	11.2	7.7	7.2
										4.3					
	FAO Reference protein pattern	...	...	...	...	...	4.2	1.4	2.8	2.2 2.0		2.8	4.8	4.2	4.2
B <sub>7</sub>	Groundnut 80 parts	...	...	...						4.2					
	+ Skim milk powder 20 parts	...	...	...	10.2	2.2	3.9	1.1	5.2	1.4 1.5		2.0	7.3	5.0	4.8
										2.9					
B <sub>8</sub>	Bengal gram 80 parts + Skim milk powder 20 parts	...	...	...	6.2	2.4	7.0	0.8	5.1	2.1 0.9		4.5	8.7	6.4	5.8
B <sub>9</sub>	Groundnut 60 parts + Bengal gram 20 parts + Skim milk powder 20 parts	...	...	...	9.6	2.2	4.4	1.0	5.1	1.5 1.4		2.5	7.6	5.2	4.9
B <sub>10</sub>	Groundnut 25 parts + Soyabean 30 parts + Bengal gram 25 parts + Skim milk powder 20 parts	...	...	...	8.0	2.5	5.9	1.2	5.3	1.6 0.9		3.4	8.1	5.8	5.3
B <sub>11</sub>	Groundnut 25 parts + Sesame 10 parts + Soyabean 30 parts + Bengal gram 25 parts + Skim milk powder 10 parts	...	...	...	8.4	2.5	5.3	1.2	5.5	1.7 0.9		3.4	7.7	5.6	5.2
B <sub>12</sub>	Groundnut 30 parts + Sesame 10 parts + Soyabean 30 parts + Bengal gram 30 parts...	...	...	...	9.0	2.4	5.5	1.2	5.5	1.5 1.0		3.2	7.5	5.4	5.0

The sample of rice flour was obtained by powdering a sample of raw milled rice (*Var.-Bangaru sanna*) in a flour mill.

Skim milk powder (Mafco brand) was used in the investigation. The essential amino acid composition of the proteins of samples of different foodstuffs were determined by the methods adopted by Tasker *et al*<sup>14</sup>. The data were used for the calculation of the amino acid composition of different protein blends.

Blends of soyabean flour, sesame flour, skim milk powder, rice flour and groundnut flour were prepared. The amino acid composition of the five blends (B<sub>2</sub> to B<sub>5</sub>) was nearly the same as that of FAO reference protein pattern. The remaining blends (B<sub>6</sub> to B<sub>12</sub>) had varying amino acid compositions. The amino acid composition of the different blends is given in Table I. The blend of soyabean flour and groundnut flour was fortified with



TABLE II  
Nutritive value of vegetable protein blends having an amino acid composition similar to the FAO reference protein pattern  
(6 males and 6 females in each group) (Level of protein: 10%)

Sl. No.	Blend No.	Diet	Protein contributed %	Protein intake (g)		Gain in weight (g)		PER		
				4 weeks	8 weeks	4 weeks	8 weeks	4 weeks	8 weeks	
1	B <sub>1</sub>	Soyabean	...	6	27.09	61.59	60.8	127.8	2.24	2.06
		Sesame meal	...	4						
2	B <sub>2</sub>	Rice	...	6	23.99	53.75	57.5	118.2	2.39	2.19
		Skim milk powder	...	4						
3	B <sub>3</sub>	Skim milk powder	...	5	23.02	55.57	56.4	123.3	2.44	2.20
		Sesame	...	5						
4	B <sub>4</sub>	Groundnut	...	5	27.00	57.90	47.6	95.7	1.76	1.66
		Soyabean	...	5						
5	B <sub>5</sub>	Groundnut	...	5	25.88	60.03	55.1	117.5	2.12	1.96
		Soyabean + methionine	...	5						
6	B <sub>6</sub>	INCAP 9B	...	10	24.67	57.87	48.6	98.8	1.97	1.70
7	...	Skim milk powder	...	10	23.14	59.58	69.9	143.4	3.02	2.40

Standard error of the mean	± 0.06 (60 d.f.)	± 0.05 (60 d.f.)
Critical difference at 5% level	0.17	0.14
„ „ 1% „	0.23	0.18
„ „ 0.1% „	0.30	0.24

methionine so as to bring the cystine-methionine content to that of FAO reference protein pattern. For comparison, INCAP mixture 9B developed by Scrimshaw *et al*<sup>10</sup>. was included in the present study. Other blends included in this study were soya flour fortified with methionine, groundnut flour fortified with lysine, methionine and threonine and a mixture of groundnut flour, soyabean flour and Bengal gram flour fortified with methionine.

*Determination of protein efficiency ratio:* The protein efficiency ratios of the individual proteins and protein mixtures at 10 per cent level of protein intake were determined by the rat growth method of Osborne, *et al*<sup>15</sup>. Weanling albino rats (28 days old) weighing 40-43 g from the laboratory stock colony were allotted to different groups according to simple randomized block design ignoring litters. The protein content in all the diets was maintained at approximately 10 per cent level on dry weight basis, the protein being derived from one of the blends. The diets were made adequate with respect to other nutrients<sup>11</sup>. The diets were cooked with three times the weight of water by steaming for 10 minutes and fed *ad lib* to the rats. Records of food intake were maintained for each animal. The rats were weighed weekly.

Results and discussion

*PER of protein blends having amino acid composition similar to that of FAO reference protein pattern:* The results given in Table I show that the amino acid composition of blends B<sub>2</sub>, B<sub>3</sub> and B<sub>5</sub> is closely similar, while that of blends, B<sub>1</sub> and B<sub>4</sub> differs slightly from that of FAO reference protein pattern. Table II shows that the PER of the different blends ranged from 2.12 to 2.44 (in 4 weeks) and from 1.96 to 2.2 (in 8 weeks) and were significantly



TABLE III (a)

Effect of incorporation of skim milk powder on the nutritive value of blends of vegetable proteins  
(6 males and 6 females in each group) (Level of protein: 10%)

Sl. No.	Blend No.	Source of protein in diet	Protein contributed %	Protein intake (g)		Gain in weight (g)		PER	
				4 weeks	8 weeks	4 weeks	8 weeks	4 weeks	8 weeks
1	B <sub>7</sub>	Groundnut flour	...	8.5					
		Skim milk powder	...	1.5	19.54	43.43	45.8	83.3	2.34
2	B <sub>8</sub>	Bengal gram flour	...	7					
		Skim milk powder	...	3	15.33	38.26	35.2	69.5	2.28
3	B <sub>9</sub>	Groundnut flour	...	7.2					
		Bengal gram flour	...	1.1	19.21	43.66	45.8	89.7	2.39
		Skim milk powder	...	1.7					
4		Skim milk powder	...	10	20.59	43.35	60.4	97.4	2.93
									2.26

Standard error of the mean	± 0.08 (30 d.f.)	± 0.06 (30 d.f.)
Critical difference at 5% level	0.23	0.17
“ “ 1% “	0.31	0.23
“ “ 0.1% “	0.41	0.30

less than that of the proteins of skim milk powder (3.02 and 2.40 in 4 and 8 weeks). This is evidently due to the fact that the different protein blends as well as FAO reference protein pattern did not provide the optimum amino acid requirements of the rat<sup>16</sup>. It is also of interest to note that the PER of INCAP formula 9B were only 1.97 and 1.70 (in 4 and 8 weeks). This blend has, however, been reported to meet the protein requirements of even children suffering from kwashiorkor<sup>10</sup>.

*PER of vegetable protein blends containing 20 per cent of skim milk powder:* The results presented in Table III a and b show that the PER of the different protein blends containing 20 per cent skim milk powder ranged from 2.25 to 2.39 (in 4 weeks) and 1.82 to 2.05 (in 8 weeks) and were of the same order as those obtained for blends having amino acid composition similar to that of FAO reference protein pattern (Table III). The PER of the blends were slightly higher than that of INCAP formula 9B but significantly lower than that of the proteins of skim milk powder. It is of interest to note that protein blends B<sub>9</sub> (consisting of 60 parts of groundnut flour, 20 parts of Bengal gram flour and 20 parts of skim milk powder) and B<sub>8</sub> (consisting of 80 parts of Bengal gram flour and 20 parts of skim milk powder) have been found to be effective for the treatment of protein malnutrition in children<sup>9, 17</sup>.

*Effect of fortification of soyabean and groundnut flours with limiting amino acids on the PER:* Table IV shows that the PER of the proteins of soya bean flour fortified with DL-methionine (2.89—2.98 in 4 weeks) and (2.30—2.38 in 8 weeks), are of the same order as that of the proteins of skim milk powder. This finding is of great importance as DL-methionine is available in large quantities at low cost. Hence the use of soya flour fortified with DL-methionine in child feeding holds great promise. The multiple deficiency of amino acids in groundnut has been demonstrated by Macheboeuf *et al*<sup>18</sup>. and Balasundaram



TABLE III (b)  
Effect of incorporation of skim milk powder on the nutritive value of blends of vegetable proteins  
(6 males and 6 females in each group)  
(Level of protein : 10%)

Sl. No.	Blend No.	Source of protein in the diet	Protein contributed %	Protein intake (g)		Gain in weight (g)		PER	
				4 weeks	8 weeks	4 weeks	8 weeks	4 weeks	8 weeks
1	B <sub>10</sub>	Groundnut flour ...	3.3	22.20	52.47	52.4	95.5	2.36	1.82
		Soyabean flour ...	3.3						
		Bengal gram flour...	1.5						
		Skim milk powder	1.9						
2	B <sub>11</sub>	Groundnut flour ...	3.4	22.92	53.53	53.4	98.5	2.33	1.84
		Sesame flour ...	0.9						
		Soyabean flour ...	3.2						
		Bengal gram flour	1.5						
		Skim milk powder	1.0						
3	B <sub>12</sub>	Groundnut flour ...	4.0	24.84	53.62	55.9	97.6	2.25	1.82
		Sesame flour ...	0.9						
		Soyabean flour ...	3.3						
		Bengal gram flour...	1.8						
4	B <sub>8</sub>	Bengal gram flour	7	24.32	56.02	56.9	101.4	2.34	1.81
		Skim milk powder	3						
5		Skim milk powder	...	22.75	54.93	67.8	120.3	2.98	2.19

Standard error of the mean	± 0.08 (28 d.f.) ± 0.05 (28 d.f.)	
Critical difference at 5% level	0.22	0.14
„ „ 1% level	0.29	0.19
„ „ 0.1% „	0.39	0.25

TABLE IV  
Effect of supplementation with synthetic amino acids on the nutritive value of vegetable proteins

						PER 4 weeks		PER 8 weeks	
Soyabean flour	...	...	...	...	...	2.08	} ± 0.07 (80 d.f.)	1.82	} ± 0.07 (80 d.f.)
„ „ + 0.6% Methionine	...	...	...	...	...	2.89		2.30	
„ „ + 1.2% Methionine	...	...	...	...	...	2.98		2.33	
Groundnut flour	...	...	...	...	...	1.65		1.49	
„ „ + Lysine (1.5%) + Methionine (1.0%)	...	...	...	...	...	2.07		1.79	
„ „ + Lysine (1.5%) + Methio- nine (1.0 %) + Threonine (1.0%)	}			...	...	2.59		2.06	

et al<sup>19</sup>. It is also of interest to note that fortification of groundnut flour with *L*-lysine, *DL*-methionine and *L*-threonine made up for the deficiencies of different amino acids in groundnut and caused a highly significant increase in the PER. These investigations have clearly shown that it is possible to improve the nutritive value of the vegetable proteins to a considerable extent by fortification with the deficient amino acids.



## Summary

(1) The PER (at 10 per cent level) of protein blends having amino acid composition similar to that of FAO reference protein pattern ranged from 2.12 to 2.44 (in 4 weeks) and 1.96 to 2.2 (in 8 weeks) as compared with 3.02 and 2.40 (in 4 and 8 weeks) for skim milk proteins and 1.97 and 1.70 (in 4 and 8 weeks) for INCAP formula 9B of Scrimshaw *et al*<sup>10</sup>.

(2) The PER (at 10 per cent level) of blends of groundnut, Bengal gram (*Cicer arietinum*) and soyabean, containing 20 per cent skim milk powder, ranged from 2.25 to 2.39 (in 4 weeks) and 1.82 to 2.05 (in 8 weeks).

(3) Fortification of soyabean flour with *DL*-methionine (at 0.6 and 1.2 per cent levels) increased the PER of the proteins to 2.9 and 2.4 (in 4 and 8 weeks) i.e. almost to the same level as that of milk proteins, while fortification of groundnut flour with *L*-lysine, *DL*-methionine and *L*-threonine increased the PER to 2.6 and 2.1 (in 4 and 8 weeks).

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# STUDIES IN NUTRITIVE VALUE OF DOUBLE BEAN (*VICIA FABA*, MOENCH)

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Double bean is reported to be commonly consumed in the southern part of the country but no systematic work regarding its nutritive value is reported in the literature. Sohonie and Bhandarkar<sup>1</sup> reported the presence of a trypsin inhibitor in double bean. Sohoni and Ambe<sup>2</sup> isolated and purified the double bean trypsin inhibitor and studied some of its properties. Sohonie *et al.*<sup>3</sup> studied the effect of feeding double bean trypsin inhibitor and raw double bean on the growth of rats. Purified trypsin inhibitor fed at 0.265 per cent level along with a synthetic casein diet had no deleterious effect on the growth of rats. Raw double bean diet had a deleterious effect on the growth of rats whereas better growth was observed in animals fed on double bean autoclaved for half an hour at 15 lb pressure. Supplementation of the diet with methionine, in which double bean protein is deficient, had beneficial effect only when it was incorporated in the diet which contained double bean autoclaved for half an hour. From these studies it was felt that it would be of considerable interest to study the nutritive value of double bean and nature of the factor which may be responsible for causing the growth depression in rats. The work on these two aspects will be dealt with separately.

## Biological studies

Nutritive value of raw double bean and autoclaved double bean supplemented with essential amino acids.

*Experimental procedure:* Experiments were conducted on young male albino rats to study the biological value of double bean as determined by the growth method of Osborne, Mendel and Ferry<sup>4</sup>.

In all the diets 10 per cent level of protein was used. The composition of the diet was as suggested by Liener<sup>5</sup>: proteins 10 per cent, sugar 31.2 per cent, salt mixture 4 per cent, groundnut oil 6 per cent and the total was made to 100 by the addition of corn starch. The salt mixture had the composition recommended by Hegsted<sup>6</sup>. The vitamin mixture had the composition suggested by Schultz<sup>7</sup>. Vitamin A 1,200 I.U. and vitamin D 200 I.U. was also fed to each rat along with the diet.

The rats were kept in individual cages with round bottoms. Water was available *ad libitum*. In order to avoid unequal intake of diet causing excessive gain or loss in weight of the animals, pair feeding technique was used.

*Results and discussion:* In the case of a variety of legumes it was observed that the nutritive quality could be improved by the supplementation of the diet with essential amino acids in which they are markedly deficient. Sohonie *et al.*<sup>3</sup> observed that when raw double bean diet was supplemented with methionine it had no beneficial effect on the growth of rats. It was, therefore, felt it would be interesting to study the effect of



TABLE I

*Essential amino acid composition of double bean casein and the minimum essential amino acid requirement of the rat*

Amino acid			Casein	Double bean	Essential amino acid requirement for rat (% of diet)
			g/16g nitrogen		
Arginine	...	...	5.0	9.14	0.2%
Histidine	...	...	2.15	1.4	0.4%
Leucine	...	...	11.3	7.93	0.9%
Cystine	...	...	0.3	1.41	...
Methionine	...	...	3.7	0.67	0.6%
Isoleucine	...	...	7.05	6.59	0.5%
Valine ...	...	...	7.53	3.21	0.7%
Phenylalanine	...	...	5.30	5.30	0.7%
Tryptophan	...	...	2.24	0.125	0.2%
Lysine ...	...	...	7.8	5.3	1.0%

supplementing raw double bean diet with other essential amino acids in which double bean is deficient. From the amino acid composition of double bean presented in Table I it can be seen that double bean is markedly deficient in valine, leucine and tryptophan along with methionine. An experiment was conducted with raw double bean and double bean autoclaved for half an hour was supplemented with these four amino acids. It can be seen from the Table II that supplementation of methionine, valine, leucine and tryptophan to the raw double bean diet had no beneficial effect but caused further promotion in growth in the case of the autoclaved sample.

Even in this case the growth promotion was not as high as with casein. These results indicated that the deficiency of essential amino acids may not be responsible for the depression in the growth of rats. In order to lend further support to this finding an experiment was conducted on rats in which the raw double bean diet was supplemented with all the amino acids in which double bean is deficient. While supplementing the diet with all the amino acids, the final concentration of all the amino acids was brought to the same level as that of the casein diet. The results of the experiment, presented in Table II, show that supplementing raw double bean diet, with all the essential amino acids has no beneficial effect. It was concluded that the deficiency of amino acids alone may not be responsible for the low nutritive value of double bean.

Fisher and Dewey<sup>8</sup> carried out experiments in which raw soyabean protein at 15 per cent level was supplemented with an essential amino acid mixture. This diet when fed to chicks overcame nearly all the growth depressing activity of the raw meal. They concluded that the growth depressing activity was due to the unavailability of certain amino acids. In order to find out whether amino acid availability is the cause of the growth depressing activity exhibited by raw double bean diet an experiment was conducted on rats where raw double bean diet was supplemented with all the amino acids at optimum levels as suggested by Rose<sup>9</sup>.

The results presented in Table II show that though this diet causes growth promotion when fed to rats it is still inferior to casein even though the total amino acid content of this diet is more than that of casein. It appears that the deficiency of amino



TABLE II  
*Effect of supplementing double bean diet with deficient amino acids  
on the growth of rats (pair-fed)*

Dietary group	Initial wt (g)	Change in wt after 4 weeks (g)	Diet consumed (g rat)
EXPERIMENT I—Effects of supplementation of raw double bean diet with methionine, valine and tryptophan.			
Casein diet ... ..	35.8	$25.0 \pm 1.7$	117
Raw double bean diet ... ..	35.8	$-8.6 \pm 2.0$	
Raw double bean diet supplemented with amino acids ... ..	35.3	$-2.5 \pm 1.0$	
EXPERIMENT II—Effect of supplementing double bean diet autoclaved for half an hour with methionine, valine, leucine and tryptophan.			
Casein ... ..	41.0	$31.2 \pm 2.3$	171.5
Double bean autoclaved... ..	41.6	$12.4 \pm 2.7$	
Double bean autoclaved supplemented with amino acids ... ..	41.4	$22.2 \pm 2.5$	
EXPERIMENT III—Effect of supplementing all the amino acids in which double bean is deficient.			
Casein diet ... ..	45.2	$27.2 \pm 2.7$	119.6
Raw double bean diet ... ..	46.2	$-13.8 \pm 2.2$	
Raw double bean diet supplementing all the amino acids ... ..	45.6	$-45.6 \pm 2.5$	
EXPERIMENT IV—Effect of supplementing raw double bean diet with all the essential amino acids at optimum levels.			
Casein ... ..	44.5	$14.8 \pm 1.1$	110.0
Raw double bean diet ... ..	44.5	$-14.5 \pm 3.0$	
Raw double bean diet supplemented with all the amino acids at optimum levels ... ..	44.3	$-7.5 \pm 2.6$	
EXPERIMENT V—Effect of feeding trypsin (2%) level along with raw double bean diet.			
Casein ... ..	38.5	$18.4 \pm 1.0$	118.0
Raw double bean ... ..	38.7	$-9.7 \pm 1.0$	
Raw double bean trypsin ... ..	38.6	$-8.4 \pm 1.0$	

acids may not be responsible for causing growth depression when raw double bean is fed to rats as a sole source of protein but the presence of an anti-growth factor rendering the essential amino acids non-available to the animal might be responsible for the growth inhibition.

Almquist and Merritt<sup>10,11</sup> reported that crude trypsin when added at a sufficiently high level to raw soyabean diet overcomes the growth depressing activity when fed to chicks.



An experiment conducted to find out whether addition of crude trypsin at 2 per cent level to raw double bean diet causes beneficial effect showed that the nutritive quality is not improved (Table II).

From these biological studies it appears that growth inhibition in rats when fed on raw double bean diet is not due to the deficiency of essential amino acids and trypsin inhibitor present in double bean but due to the presence of a toxic or antigrowth factor which appears to affect the availability of essential amino acids.

### Studies on the antigrowth factor

(a) *Chemical studies*: It is well known that certain foodstuffs contain some factors which are either growth promoting or growth depressing. Dupuy and Lee<sup>12</sup> reported that  $\beta$ -amino-propionitrile which is present in certain varieties of *Lathyrus sativus* seeds causes Lathyrism. Miller<sup>13</sup> reported that dihydroxyphenylalanine present in velvet beans causes deleterious effects. Morris and Pagan<sup>14</sup> reported that  $\beta$ -nitropropionic acid present in creeping indigo was toxic to chicks and other animals. Appel<sup>15</sup> showed that canavanine present in jack bean produced symptoms of intoxication when injected to dogs in a dose of 200-400 mg/kilo body weight. Elliot<sup>16</sup> has reviewed the physiological action exhibited by  $\gamma$ -amino butyric acid.

In view of the evidence cited, raw double bean flour was tested for the presence of dihydroxyphenylalanine, canvanine,  $\beta$ -nitropropionic acid,  $\beta$ -amino-propionitrile and  $\gamma$ -aminobutyric acid by chromatographic procedures. These known toxic substances were absent in raw double bean meal.

(b) *Studies on isolated double bean protein*: Osborne and Campbell<sup>17</sup> reported that the protein fraction of soyabean—glycinine—was capable of supporting the growth of rats, though, the unheated soyabean meal was an unsatisfactory source of protein. De and Ganguli<sup>18</sup> also reported similar observations. Liener<sup>19</sup> concluded that the low nutritive value of unheated soyabean was due to some component of soyabean protein other than the major component.

From the literature cited above it was felt worthwhile to study the effect of feeding isolated double bean protein on the growth of rats.

The double bean protein was isolated by acetone precipitation of the water extract of double bean flour<sup>20</sup>.

The acid hydrolysates of casein and double bean protein were prepared by treating the protein with  $\text{H}_2\text{SO}_4$  for 14 hours at 15 lb pressure. The pH was adjusted to 5 with barium hydroxide. Tryptophan was added to the hydrolysates as it is destroyed during hydrolysis. The enzymatic hydrolysates of isolated double bean protein and casein were prepared by treating it with trypsin at pH 8 (37°C).

The following two animal experiments were conducted on young male albino rats using the pair-feeding technique: (a) effect of feeding isolated double bean protein to rats, with or without supplementation of methionine, valine, leucine and tryptophan and (b) the effect of feeding isolated double bean protein, acid and enzymatic hydrolysates to rats, with or without supplementation of methionine.

From the results presented in Table II it can be seen that isolated double bean protein when fed to rats at 10 per cent protein level was slightly more toxic as compared with the raw double bean. After supplementing it with those amino acids in which double bean



TABLE III

*Growth promoting value of isolated double bean protein and its acid and enzymic hydrolysates*

Dietary group	Initial wt g	Change in wt after 4 weeks g
EXPERIMENT VI—Effect of feeding isolated double bean protein on the growth of rats.		
Casein	44.5	19.6
Raw double bean	45.6	— 12.0
Isolated double bean protein	44.4	— 16.6
Isolated double bean protein supplemented with amino acids	45.0	— 7.0
EXPERIMENT VIII—Effect of feeding double bean protein, acid enzymatic hydrolysates on the growth of rats.		
Casein (acid hydrolysates)	44.6	14.0
Double bean protein (acid hydrolysate)	44.6	— 10.0
Double bean protein (acid hydrolysate) supplemented with methionine	45.0	12.7
Casein (enzymatic hydrolysate)	45.0	19.6
Double bean protein (enzymatic hydrolysate)	44.5	— 6.5
Double bean protein (enzymatic hydrolysate) supplemented with methionine	44.5	— 9.5

protein is deficient, no growth promotion in rats was observed. It appeared that double bean protein might contain a toxic component which inhibits the growth of rats.

When the results of feeding acid hydrolysates of isolated double bean protein presented in Table III are compared with those of casein it can be seen that the isolated double bean protein acid hydrolysate does not support the growth of rats. The decrease in weight of the animals is similar to that observed when raw double bean or double bean protein is fed to rats as the sole source of protein. Supplementation of double bean protein acid hydrolysate with methionine had a beneficial effect on the growth of rats.

When the results of feeding enzymatic hydrolysates of casein are compared with those of double bean protein it can be seen that the enzymatic hydrolysate of casein supports the growth of rats while that of double bean protein inhibits the growth and the degree of inhibition is similar to that observed when raw double bean or double bean protein is fed to rats.

Though the enzymic hydrolysate of casein was superior to the acid hydrolysate, no such relation was observed when the results of feeding acid and enzymic hydrolysates of double bean protein were compared. However, in the case of acid hydrolysates supplementation with methionine helped to improve the nutritive quality. In the case of enzymatic hydrolysates no such improvement was noticed.

The results showed that the deleterious effects of feeding double bean protein could be removed by hydrolysis with acid and not by enzymatic hydrolysis.

From these experiments it appeared that:

1. Double bean proteins are of inferior quality, being deficient in several essential amino acids.
2. Supplementation of deficient amino acids does not improve the nutritive quality.



3. A growth inhibitor is present which may be a protein in nature or very closely associated with protein, which renders the essential amino acids non-available to the animals.

## Summary

Experiments were conducted on young male albino rats to study the nutritive value of double bean. Double bean was found to cause deleterious effects on the growth of rats. Supplementation of essential amino acids in which double bean is deficient had no beneficial effect. Further studies with isolated double bean protein showed that a growth inhibitor is present which may be protein in nature or very closely associated with protein.

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## Discussion

- Q. Is the antigrowth factor identical with the trypsin inhibitor in the bean?
- A. The isolated and purified trypsin inhibitor had no deleterious effect on the growth of rats when fed along with a synthetic casein diet. Hence the two factors must be different.



# CLINICAL TRIALS WITH VEGETABLE PROTEIN FOODS

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## Introduction

Protein malnutrition in children is primarily due to a deficiency of protein in the diet during the critical weaning and post-weaning period when the requirement of this nutrient is high. Kwashiorkor, one of the extreme manifestations of protein deficiency in children has been recorded to occur frequently among Indian children in different parts of the country<sup>2</sup>. There is no doubt that kwashiorkor, using the term in its widest sense, is the outstanding pediatric nutritional problem in our country to-day. Indeed it could be classed as a vast medico-social problem since clinical survey of the child population in the country has revealed the existence of a large number of children with underlying protein deficiency constantly exposed to the risk of the development of the full blown disease<sup>3</sup>.

The treatment of kwashiorkor in the clinic consists of providing protein of good biological value in an easily assimilable form. Reconstituted skimmed milk in suitable dilutions has proved to be very effective in rapidly controlling the clinical manifestations of the disease<sup>3</sup>. While the administration of skimmed milk is the ideal method of treatment of patients in the hospital wards, under the present circumstances and in the immediate future skimmed milk cannot be the practical answer to the problem of widespread protein deficiency in the country. This raises the question whether protein deficiency in childhood could be corrected or prevented by using proteins from vegetable sources. Since the present need is a solution and not a palliative it is imperative that cheap sources of vegetable proteins have to be found for the control of the problem.

While several factors have to be taken into consideration in the choice of the vegetable protein food, the most important and possibly the final test of suitability is the capacity of the vegetable protein food to control the clinical manifestations of severe protein malnutrition. Clinical trials with different vegetable protein foods were, therefore, undertaken in the Nutrition Research Laboratories during the last few years to test their efficacy in the treatment of kwashiorkor<sup>4,5</sup>.

## Materials and Methods

*Clinical material:* Two hundred and twenty one cases of kwashiorkor formed the subject of this investigation. The clinical features and age distribution of the patients were the same as those described earlier<sup>1</sup>. The patients were admitted in the nutrition ward of the hospital and were under daily observation for a period of 30-45 days.

*Diets:* Five different types of vegetable protein 'foods' were investigated. The response obtained with the vegetable proteins was compared with that obtained using skimmed milk as a source of protein in another group. The composition of the foods,



TABLE I

*Composition and nutritive value of the diets*

Diet	Main source of protein	Composition	Nutritive value			No. of patients on the diet
			Protein g	Calories	Calcium mg	
1	Skimmed milk powder ...	Skimmed milk powder 140 g Bakery bread 224 g Jaggery 28 g	63	1,100	2,000	63
2	Single pulse ...	Bengal gram powder 250 g Peeled ripe banana 100 g Jaggery 70 g	60	1,475	254	56
3	Pulse-cereal ...	Bengal gram powder 190 g Rice powder 250 g Sodium chloride 3 g Calcium lactate 3 g	61	1,677	552	19
4	Pulse-peanut (Mysore formula A) ...	Roasted Bengal gram powder-25 parts; low fat peanuts-75 parts; calcium phosphate-1 part; lucerne-1 part } 140 g Bakery 170 g Jaggery 57 g	70	1,425	1,138	21
5	Pulse-peanut sesame (Mysore formula B) ...	Roasted Bengal gram powder-25 parts; low fat peanut-49 parts; low fat sesame-25 parts; calcium phosphate-1 part; lucerne-1 part } 150 g Bakery bread 170 g Jaggery 70 g	70	1,430	1,625	41
5a	„	Mysore-formula B 270 g Jaggery 85 g	100	1,340	2,925	10
6	Peanut ...	Defatted peanut flour-99 parts 116 g and lucerne-1 part Peeled ripe bananas 100 g Bakery bread 200 g Jaggery 70 g	70	1,380	1,260	11

their nutritive values and the number of subjects receiving the different treatments are indicated in Table I. It will be seen that clinical trials were conducted both with single as well as mixed plant protein diets. The latter were tried since vegetable proteins are deficient in one or more essential amino acids and there is the possibility of a mutual supplementation of deficient amino acids if a vegetable protein mixture is used.

*Preparation of the diets:* The vegetable protein foods were prepared in one of two forms. *Cakes:* The ingredients were mixed into a paste with suitable additions of water and autoclaved for 30 minutes at 15 pounds pressure. The autoclaving was done purely for convenience and ease of cooking. The cooked diet had a cake like appearance and consistency. *Porridge:* Some of the foods were also prepared as a porridge by mixing the



ingredients into a paste with warm water and jaggery syrup and cooking over low heat for 10-15 minutes.

The dry skimmed milk powder was reconstituted with six volumes of water into fluid milk. In 4 out of 6 groups (Table I) a fixed quantity of bakery bread was provided to increase the intake of calories.

*Method of assessment:* A complete clinical examination was carried out at the time of admission and the patients were kept under observation throughout the study. An initial sample of blood was drawn from each patient for the determination of total serum protein, serum albumin (micro Kjeldahl method), haemoglobin and serum calcium (modification of Kramer and Tisdall method—Clark and Collip<sup>6</sup>). The albumin fractionation was carried out using a 28 per cent sodium sulphite solution<sup>7</sup>. On the 10th, 30th and in some patients, 45th day of treatment with the above diets, samples of blood were again drawn for repetition of the above biochemical investigations. The clinical progress of the patients and their body weight were recorded daily.

The response obtained in the patients using the different therapeutic diets was assessed with the help of the following criteria: (a) the number of days taken for the clinical disappearance of edema, (b) the time taken for reaching the minimum body weight, (c) the time taken for rise in body weight by one pound—calculated from the day on which minimum weight was reached, (d) the time taken for the diarrhoea to subside in patients who had non-infective diarrhoea at the time of admission, and (e) the rise in plasma albumin on the 10th and 30th day of treatment. Some of the draw-backs of the above criteria have been described earlier<sup>4</sup>.

It is possible that the above criteria may be insufficient to discern the differences in the response to different diets particularly as the patients were subsisting on a severely protein deficient diet prior to their admission to the hospital. This limitation had to be kept in mind while interpreting the results of the study.

## Results

*Acceptability of the product:* No difficulty was encountered in administering the diets to the majority of patients. This was particularly true after the first couple of days in the hospital and in the case of diets which were sweetened with jaggery. The initial reluctance on the part of some of the children to consume the diet was most probably due to the anorexia and was overcome with tact, patience and perseverance. However, in the case of diet 3—pulse-cereal diet—which was very bulky, it was not possible always to feed the diet completely everyday.

*The clinical response:* The results are presented in Table II. The data indicate that the patients receiving the six different lines of high protein therapy behaved similarly as regards loss of edema fluid. It was also noted that the improvement in the associated signs of protein malnutrition, viz.—the crazy pavement dermatosis, other varieties of skin lesions, the oro-lingual signs of vitamin B complex deficiency—was equally rapid in all the groups. However, it was interesting to note that patients receiving diet 6—groundnut-protein diet—took nearly twice as long as those in the other groups to gain one pound of body weight after reaching the minimum body weight. While the vegetable protein diets helped to control the loose stools in those suffering from non-infective diarrhoea at the time



TABLE II

*Response to different lines of treatment*

Diet	Time for disappearance of edema (days)		Time for minimum weight to be reached (days)		Time for gaining one pound body weight (days)		Time for control of diarrhoea (days)		Rise in serum calcium on 30th day mg/100 ml	Rise in serum albumin g/100 ml		
	Mean	Range	Mean	Range	Mean	Range	Mean	Range		10th day	30th day	45th day
1	12	3-33	7.3	0-24	5.4	2-18	10.5	2-23	2.25*	0.75	1.24	...
2	13	4-45	9.5	0-51	5.8	1-18	5.7	2-13	...	0.40	1.04	...
3	17	4-68	14.5	0-64	5.2	3-8	8.0	3-19	...	0.20	0.63	...
4	13	4-22	7.1	2-18	7.4	1-30	7.8	2-27	1.1	0.14	0.86	...
5	13	2-30	8.6	3-21	6.1	1-17	6.3	2-16	1.3	0.12	0.76	1.06†
5 a	...	...	...	...	...	...	...	...	...	0.20	0.99	...
6	13	2-30	7.7	1-19	14.0	7-19	5.9	2-10	1.1	-0.28	0.35	...

\* from 14 subjects only.

† from 10 subjects only.

of admission, it was observed that skimmed milk aggravated or actually precipitated a diarrhoea. However, the latter was of no consequence because this undesirable response passed off with continued treatment.

It was the clinical impression that at the end of a month's stay in the hospital, all the children irrespective of the treatment group were practically fit and 'normal' except for their very low body weight.

*Changes in serum calcium:* All the patients showed at the time of admission sub-normal level of serum calcium. The low levels of serum calcium was partly the result of a low diet intake and partly an attribute of the low serum proteins. The mean increase in serum calcium on the 30th day of treatment was of the same order in patients receiving diets 4 and 5. However, this increase was insufficient to bring about a normal level of serum calcium and was only about half of that observed in patients receiving reconstituted skimmed milk (diet 1). The greater rise in serum calcium observed in the skimmed milk group is probably dependent on the greater rise in serum proteins observed among them.

*Serum albumin regeneration:* The initial serum albumin in the patients ranged between 0.9-4.2 g per cent and the mean serum albumin concentration at the time of admission in the different groups was 2.12 g, 2.78 g, 2.50 g, 2.63 g, 2.60g, 2.54 g, per cent respectively. The mean increase in serum albumin on the 10th and 30th days of treatment was highest in the patients receiving skimmed milk protein (diet 1). In the patients receiving the Bengal gram protein (diet 2) the mean serum albumin regeneration on the 10th day of treatment was only about half of that observed in the skimmed milk group. This difference, however, narrowed down with continued treatment and on the 30th day the increase was nearly 85 per cent of that recorded in the skimmed milk group.

On the other hand patients receiving the mixed vegetable protein diets 3, 4 and 5 recorded only 16 to 25 per cent mean rise in the serum albumin level on the 10th



day of treatment and 50 to 70 per cent rise on the 30th day compared to those who received skimmed milk protein. Indeed in some of the children treated with diets 4 and 5 there was an actual fall in serum albumin on the 10th day of observation. While the inferiority of vegetable proteins in general to skimmed milk protein in this particular respect was not unexpected, the negligible rise observed with mixed vegetable proteins in comparison to that achieved through single pulse protein (diet 2) was inexplicable. In a few patients where the treatment with one of the mixed vegetable proteins (diet 4) was continued beyond the 30th day, it was found that the rise in serum albumin on the 45th day of therapy was nearly the same as that brought about by the Bengal gram protein diet (diet 2) on the 30th day. An increase of protein intake from 70 to 100 g supplied through diet 4 in a few patients brought about a significantly higher increase on the 30th day of treatment but without any obvious 'benefit' on the 10th day of observation. In fact half the number of patients in the latter experiment showed a reduction in the serum albumin level on the 10th day of treatment.

The group of children who received diet 6, based on defatted groundnut flour showed the poorest response in so far as the regeneration of serum albumin was concerned. 9 out of 11 patients recorded no rise or an actual drop on the 10th day of treatment. At the end of the treatment period, 5 out of 11 subjects showed no rise in serum albumin and the mean rise was only 28 per cent of that obtained in the skimmed milk group.

## Discussion

The results of this investigation proved that the vegetable protein diets used in the clinical trials were effective in controlling the acute clinical manifestations of kwashiorkor. The vegetable proteins were, however, inferior to skimmed milk so far as the increase in serum calcium and regeneration of serum albumin were concerned. Even among the vegetable proteins, the single pulse protein (Bengal gram diet 2) appeared to be superior to the mixed vegetable proteins (diets 3, 4 and 5) in its capacity to increase the serum albumin. On the basis of the results it is suggested that the inclusion of the defatted groundnut flour in diets 4 and 5 was probably responsible for the retardation in the serum albumin regeneration in the patients receiving those diets. This comparatively unsatisfactory response obtained with the defatted groundnut flour protein was all the more significant in the light of the finding that kwashiorkor babies receiving this diet took considerably longer time to gain body-weight than those fed with the other vegetable protein foods. Notwithstanding the low rate of regeneration of serum albumin observed in the groups receiving vegetable proteins, it seemed, a satisfactory total increase could be obtained either by prolonging the treatment period or by increasing the daily intake of protein from these sources. It is doubtful, however, that the latter method of increasing the intake of protein to 100 g per day is practicable since it might produce considerable digestive strain.

The factors which bring about alterations in the serum albumin concentration during the treatment of kwashiorkor have to be understood before the significance of the observation regarding the serum albumin regeneration with different diets could be assessed. Previous investigations on adult patients of nutritional edema—calorie-protein deficiency had revealed a low plasma volume which expands during rehabilitation<sup>8</sup>. It was possible, therefore, that during treatment the kwashiorkor patients would have responded with an increase in plasma volume with a concomittant reduction in serum albumin concentration.



The serum albumin concentration could also be influenced by the quality of the protein fed. In protein depleted adult rats, Phansalkar *et al.*<sup>9</sup> demonstrated a satisfactory regeneration of serum proteins using two types of vegetable protein diets. However, the authors found that the time taken in the case of vegetable protein diets was somewhat longer than with skim milk. Since liver is the site of synthesis of serum albumin and there is considerable evidence that the organ is damaged in kwashiorkor, the rate of serum albumin regeneration would depend on the rate of recovery of this function by the liver. On the basis of histological studies on the liver of young rats fed with cereal protein, pulse-cereal-leaf protein and skimmed milk protein, Sriramachari *et al.*<sup>10</sup> stated that the livers of animals on cereal protein showed on histological examination, a pattern similar to that observed in the livers of kwashiorkor babies and the liver of animals on pulse-cereal-leaf protein did not show fatty infiltration in the majority of animals. The livers of animals receiving skimmed milk protein did not show any abnormality. The third factor which could influence the magnitude of increase of serum albumin during treatment is the initial level of serum albumin itself. However, this factor need not be reckoned in the interpretation of the results because the patients in the various groups showed uniformly low levels of serum albumin at the time of admission and the mean initial serum albumin levels in the different groups were not significantly different.

On the basis of the above discussion it appears that the serum albumin level in kwashiorkor at a particular stage during recovery is determined by the two opposite factors—increase in plasma volume with resultant reduction of albumin concentration and recovery of liver function associated with a positive regeneration of serum albumin. It is possible, therefore, that with some of the vegetable protein diets the rate of serum albumin regeneration is relatively slower than the speed with which plasma volume returns to normal level. This suggestion is supported by the finding that the differences in the level of serum albumin observed with different vegetable protein diets and skimmed milk were maximal on the 10th day with a tendency to get neutralised with the progress of treatment. Increase in the intake of protein did not bestow any additional advantage to the subject in overcoming the differences observed on the 10th day of treatment with regard to serum albumin concentration. Though the present investigation revealed the existence of certain differences with regard to the speed of serum albumin increase, it was felt that the practical significance of this from the point of view of control and prevention of protein malnutrition might not be great. However, the study did bring to light the fact that inclusion of defatted groundnut flour in the vegetable protein diet made the latter inferior with respect to its ability to regenerate serum albumin. Further investigations are in progress to assess the nature of this defect in the defatted groundnut flour.

The present investigation also provided some information on the amount of protein that would be necessary per day for the treatment of cases of kwashiorkor. When two levels (70 g and 100 g) of mixed vegetable protein (diet 5) was given to two groups of patients, the group receiving 100 g of vegetable protein per day came out with a greater rise in the serum albumin level than that receiving 70 g of the vegetable protein. However, in an investigation carried out by Jayalakshmi *et al.*<sup>11</sup> using 60 g and 100 g of protein from milk in the treatment of 2 groups of cases of kwashiorkor the authors did not find any advantage in using the higher dose. The minimum amount of protein needed by patients suffering from protein malnutrition is apparently determined by the quality of the protein.



The above results have considerable significance to those engaged in the prevention of protein malnutrition in the country. Plant proteins in suitable combinations not only have therapeutic possibilities, but also offer a cheap method of preventing protein deficiency widely prevalent among our toddlers and minimise the risk of kwashiorkor developing in them. The clinical trials with vegetable protein foods should be extended to field trials with healthy children to assess their value in the prevention of protein-malnutrition. Simultaneously search must be made for better and cheaper combinations of vegetable proteins which should be rigorously tested in the laboratory and the clinic.

## Summary

The results of treatment of 158 patients suffering from kwashiorkor using five different types of vegetable protein diets have been presented. The response obtained with the vegetable protein diet was compared with that obtained in 63 patients who were treated with reconstituted skimmed milk. The vegetable protein diets used in the study appeared to be as effective as skimmed milk protein in controlling the acute clinical manifestations of kwashiorkor. Differences were observed with regard to regeneration of serum albumin between patients receiving skimmed milk and those receiving the vegetable protein diets. There were also differences in the serum protein regeneration among patients receiving different types of vegetable protein diets. The significance of these findings is discussed and the problems involved in the use of vegetable protein foods in the treatment and prevention of protein malnutrition are indicated.

The authors are grateful to Dr C. Gopalan, Deputy Director, Nutrition Research Laboratories, for his interest and guidance in the course of these investigations.

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## Discussion

Q. Have any observations been made with isolated groundnut protein ?

A. Yes, clinical trials were carried out with groundnut protein isolate supplied by the CFTRI. Preliminary results indicate that a blend of groundnut protein isolate with skim milk powder providing the proteins from the two sources in the proportion of 2:1 respectively gave as good results as with skim milk



powder. If these results are confirmed in more extensive trials, it would be a very valuable contribution as it points to a practical means of extending existing milk protein sources with a plentifully available source of vegetable protein.

- Q. Could the lower rate of regeneration of serum albumin in the vegetable proteins as contrasted with skim milk powder be ascribed to lysine deficiency ?
- A. It is known that casein promotes serum globulin regeneration and lactalbumin the regeneration of serum albumin, but there is no proof that lysine specifically enhances the latter. Our own view, as stated above, is that a rapid restoration of plasma volume with a slower recovery of liver function with particular reference to albumin synthesis may be responsible for the observed effect.
- Q. Has the effect of methionine supplementation been studied ?
- A. In some of the cases where methionine supplements were administered there was no extra-amelioration over and above that due to the proteins.
- Q. What was the effect of the protein supplements on the serum cholesterol and total lipid content ?
- A. Both were lowered.



# TREATMENT OF PROTEIN MALNUTRITION IN CHILDREN WITH A VEGETABLE PROTEIN FOOD

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During the last few years, it has been our experience that the incidence of kwashiorkor and other less dramatic states of protein deficiency has greatly increased in Hyderabad, and their management in the hospital as well as in the various peripheral pediatric centres has put a great strain on the resources of the institutions. For example, for the five year period 1954-58, nearly 16 per cent of the total pediatric admissions in the Niloufer hospital was contributed by protein malnutrition. It must be emphasized that this high figure for admission represents only the patients in serious condition and does not include the large number of children with milder signs and symptoms of protein deficiency treated in the out-patient departments. It has not always been possible for us to provide especially for the out-patient cases any skimmed milk or other protein concentrates.

The object of the present investigation was to study the response to Indian MPF (a product formulated by the Central Food Technological Research Institute, Mysore), in cases of kwashiorkor and to compare the results with that obtained using skimmed milk protein<sup>1</sup>. Since it is often stated that vegetable protein foods are inferior to animal protein foods due to the absence of vitamin B<sub>12</sub>, a third group of patients were treated with the MPF with an additional supplement of parenteral vitamin B<sub>12</sub>.

## Materials and Methods

Twenty one patients suffering from kwashiorkor were studied. All the patients satisfied the minimal criteria for the diagnosis of kwashiorkor<sup>2</sup>. The patients were observed for a total period of thirty days. The clinical improvement was recorded daily, while the haemoglobin and plasma proteins (Biuret method) were determined on admission and on the thirtieth day of treatment. A piece of liver for histological study was obtained both at the time of admission and at the end of investigations from three patients receiving skimmed milk and four receiving the MPF.

The composition, the amount and the nutritive value of the therapeutic diets are shown in Table I.

The skimmed milk powder was reconstituted as a thick suspension in 1:4 of water. The MPF was cooked and given in three different forms: (a) as porridge with added jaggery (b) as a paste spread over bread (c) and as sweet laddoos. It will be observed that bread was added to the diet of the patients in order to increase the calorie intake. Table I does not give the additional amount of protein obtained from bread, which, however, was small and equal in all the three groups.

The response to the skimmed milk and the MPF was assessed using the same criteria employed by Venkatachalam *et al*<sup>3</sup>.



TABLE I  
*Composition and nutrient content of the therapeutic diets*

Group	No. of patients	Composition	Amount (oz)	Calories	Protein (g)	Calcium (mg)
I	8	Skimmed milk powder	6.0	1,416	60	2,418
		Jaggery	2.0	...	...	...
		Bread	8.0	...	...	...
II	7	MPF mixture	5.0	...	...	...
		Jaggery	2.5	1,430	60	672
		Bread	8.0	...	...	...
III	6	Same as Group II, in addition the patients received injection of vit. B <sub>12</sub> 50 mcg. i.m. on alternate days	...	1,430	60	672

\* MPF mixture is Indian Multi-Purpose Food, Formula 'A', evolved by the C.F.T.R.I., Mysore.

## Results and Discussion

The MPF diet was found to produce frequent abdominal distension and occasionally induced vomiting. The occurrence of these side effects made the feeding a little more difficult than skimmed milk administration. Another defect noticed was the presence of a nutty flavour in the cooked product which evoked resistance from the mothers though not from the patients, since the former had the deep rooted belief that pulses are generally bad for digestion, particularly for the sick children. The non-specific diarrhoea which was observed frequently in those receiving skimmed milk was conspicuous by its absence in those fed the MPF diet.

The response of the patients to the three different lines of treatment is indicated in Figure 1 and Table II. The differences in the time taken for the clinical disappearance of oedema and attainment of minimum body weight between the three groups were slight and not statistically significant. The gain in body weight on the thirtieth day calculated from the day minimum body weight was attained ranged from 1.7 to 2.9 lb; and this difference was also not significant.

The mean haemoglobin concentration in the three groups at the end of treatment was slightly less than the mean figures at the time of admission. The lack of improvement of the anaemia on high protein therapy suggests that the anaemia was not due to a simple protein deficiency alone.

The mean increase in serum albumin in groups I and III was nearly the same. On the other hand, the mean increase in serum albumin in group II was only 1.4 g per cent, which was lower than those of group I and III. However, the difference was not statistically significant.

In the few subjects in whom liver biopsy was done before and after treatment, it was found that the fatty infiltration disappeared completely both in the skimmed milk and the MPF groups.



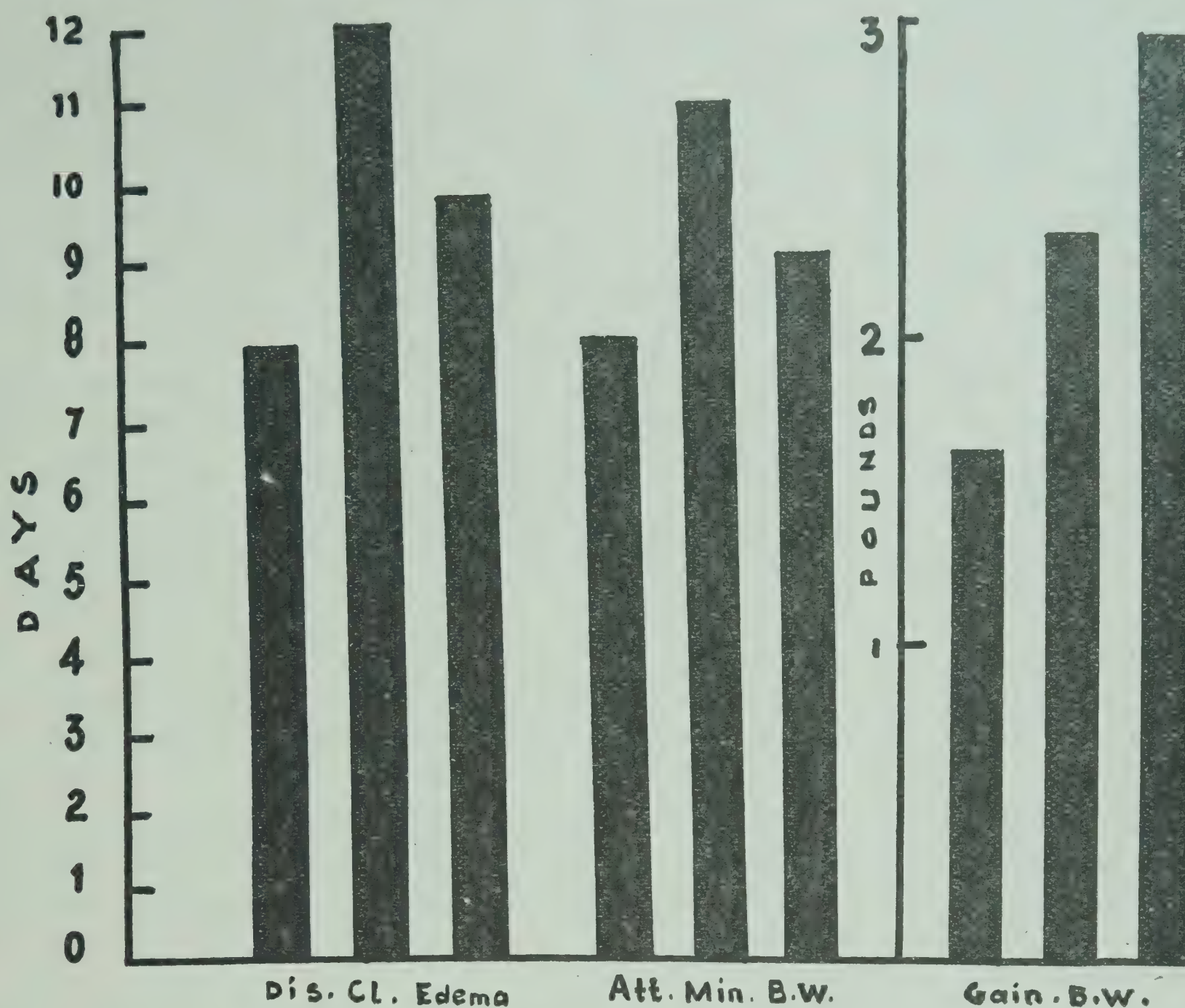


FIG. 1. Clinical response to treatment.

TABLE II  
*Haemoglobin and serum proteins before and after therapy*

Group	No. of patients	Haemoglobin g %		Loss	Total proteins g %			Serum albumin g %		
		Initial	Final		Initial	Final	Gain	Initial	Final	Gain
I	8	7.2 (6.8-8.5)	6.1 (2.6-10.7)	1.1	4.3 (2.5-5.7)	6.9 (6.0-8.7)	2.6	1.9 (1.4-3.0)	3.8 (2.8-4.6)	1.9
II	7	7.9 (5.0-10.5)	7.3 (3.8-11.0)	0.6	4.2 (3.7-5.2)	6.1 (5.2-8.0)	1.9	2.1 (1.2-2.8)	3.5 (2.0-5.2)	1.4
III	6	7.8 (5.0-9.0)	7.3 (4.5-10.0)	0.5	4.5 (3.2-6.0)	6.7 (5.0-9.0)	2.2	1.9 (1.2-2.6)	4.0 (2.5-5.0)	2.1

Figures within brackets give range

The results presented above clearly indicate that the administration of MPF is able to control the clinical manifestations of kwashiorkor. The addition of vitamin B<sub>2</sub> in no way enhanced the therapeutic value of the MPF. It is understandable from this that



MPF has got a definite role in the treatment of protein malnutrition in the hospital and the community. Though it is realised that there are several secondary factors besides protein deficiency in the diet which frequently precipitate an attack of kwashiorkor in the child, such as gastrointestinal and respiratory infections, there is no doubt that if a high protein diet is continuously provided to our protein starving toddlers, it would be possible to prevent the risk of kwashiorkor supervening. Provision of cheap proteins to the growing infant and child might be expected not only to ward off the occurrence of kwashiorkor, but also to considerably reduce the susceptibility to infectious diseases and enable proper growth and development leading on to healthy adulthood.

Further intensive investigations should be carried out both in the laboratory and in the clinic to discover many more combinations of vegetable proteins similar to MPF which could serve as satisfactory substitutes for milk which is lacking in the dietary of the post-weaning child. In the meanwhile, efforts must be made to popularise the MPF in child welfare and other centres as a safe and satisfactory protein supplement for growing children.

### Summary

The results of an investigation on the response to treatment with skimmed milk, MPF and MPF with vitamin B<sub>12</sub> in a series of twenty one cases of kwashiorkor are presented.

It was found that considerable clinical improvement and increase in serum proteins could be brought about by the MPF. The significance of the above observation with respect to treatment and prevention of protein malnutrition in the country is discussed.

### Acknowledgment

The author conveys his grateful thanks to the Director of Medical Services, Andhra Pradesh for permission to take part in the Symposium on Proteins and present the results of this investigation.

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### Discussion

Q. What was the age group of the children?

A. 1½ to 2 years.

Q. What was the protein content of the diets?

A. About 20 per cent.

Q. The diarrhoea and other untoward symptoms may have been caused by fibre and other indigestible carbohydrates associated with the protein sources employed. Would it not, therefore, be desirable and advantageous to use the protein isolates?

A. In extending our clinical trials we would certainly use the isolates particularly because of the very encouraging observations made by Dr P. S. Venkatachalam and his associates.

Q. Would folic acid supplementation have helped to control the diarrhoea?

A. The diarrhoea is of the non-specific type and does not respond to folic acid.



# TREATMENT OF PROTEIN MALNUTRITION IN CHILDREN USING BLENDS OF ISOLATED VEGETABLE PROTEINS, CASEIN AND SKIM MILK POWDER

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The foodstuffs which have been extensively used in the treatment of protein malnutrition in children are skim milk powder and casein<sup>1</sup>. In view of the shortage in the supply of the above foods in several economically underdeveloped countries, investigations have been carried out by several workers on the use of protein-rich foods of vegetable origin, e.g., oilseed meals and pulses in the treatment of kwashiorkor<sup>2</sup>. Soyabean and mixtures of Bengal gram (*Cicer arietinum*), low-fat groundnut flour, skim milk powder and sesame and cottonseed meals have been found to be effective in the treatment of mild and moderately severe cases of protein malnutrition, while severe cases have still to be treated with blends of skim milk powder and calcium caseinate<sup>1, 3-5</sup>.

Protein-rich foods of vegetable origin have the following disadvantages as compared with skim milk powder for use in the treatment of severe cases of protein malnutrition: (1) they contain indigestible carbohydrates which may swell and interfere in the digestion and absorption of proteins and (2) many of them contain odoriferous and bitter principles, which affect their palatability. These difficulties, however, can be overcome by the use of proteins isolated from oilseed meals and pulses. Isolated proteins are 2 to 4 times as concentrated as the protein source and possess a bland taste. As such they can be readily blended with skim milk powder or casein and used for the treatment of kwashiorkor. A preliminary account of the results of treatment of a small number (eight) of cases of kwashiorkor using blends of isolated vegetable proteins, casein and skim milk powder is given here.

## Experimental

Since peanut protein is lacking in certain essential amino acids, it can be used for the treatment of kwashiorkor only in conjunction with milk or other animal proteins of high biological value. Fortification of the product with the deficient essential amino acids will also help to improve the protein quality. The following blends were tested in this investigation. Since children suffering from kwashiorkor also suffer from deficiencies of potassium, calcium and certain vitamins, the subjects were given a supplement of mineral salts and vitamins.

*Blend I:* Blend I contained about 60 per cent of vegetable protein (30 per cent of peanut protein and 30 per cent of soyabean protein) and 40 per cent of protein as casein. It was fortified with l-lysine HCl (0.75g) and dl-methionine (1.96g) per 100g of the blend. The amino acid composition of the blend was comparable to that of milk proteins. The purpose of this study was to find out the value of the blend in the treatment



of severe cases of protein malnutrition. The use of such blends will effect a saving of 60 per cent of protein in the form of milk proteins.

*Blend II:* Blend II consisted of 52 parts of peanut protein and 48 parts of skim milk powder. The proportion of protein contributed by peanut and skim milk powder in the blend was 3:1. Such a blend, though inferior to milk proteins and to Blend I may be effective in the treatment of moderately severe cases of kwashiorkor and would also help to conserve milk protein.

The amino acid composition of the two blends as compared with that of milk protein and FAO 'reference protein pattern' is given in Table I. Experimental results are summarised in Table II.

TABLE I  
*Amino acid composition of protein blends as compared  
to milk protein*  
(Calculated to 16 g N)

	Protein Blend No. I	Protein Blend No. II	Cow's milk	FAO reference protein pattern
Lysine ...	7.0	4.4	7.4	4.2
Tryptophan ...	1.3	1.1	1.4	1.4
Methionine ...	4.3	1.4	2.8	2.2
Methionine + cystine...	5.3	2.8	3.9	4.2
Threonine ...	3.8	3.3	4.6	2.8
Phenylalanine ...	5.3	5.3	5.5	2.8
Leucine ...	8.3	7.9	12.1	4.8
Isoleucine ...	5.6	4.7	6.7	4.2
Valine ...	5.9	5.2	7.1	4.2

Both the protein blends were fortified with the addition of essential minerals as indicated below:

*Essential minerals per 100 g of protein blend*

Dipotassium hydrogen phosphate	...	3.5 g
Disodium hydrogen phosphate	...	2.0 g
Potassium bicarbonate	...	1.0 g
Tricalcium phosphate	...	0.5 g
Calcium carbonate	...	0.5 g

**Clinical Features and Laboratory Findings**

The subjects belonged to the age group 2-5 years. In all the cases generalised oedema was present. Four of the eight cases showed mild angular stomatitis. Four cases showed skin changes, i.e. crazy pavement dermatosis over the legs, hips and/or forearm. Two cases showed discolouration of the hair. Liver and spleen were not palpable in all the cases. Conjunctiva was pale and anaemic in all the cases.



TABLE II

*Changes in body weight and serum albumin analysis of cases of "nutritional oedema syndrome" treated with different proteins of protein foods*

Protein source	No. of cases	Age (range) years	No. of days taken for clinical disappearance of oedema		No. of days taken for a rise of 1 lb body wt calculated from the first day of reaching minimum weight		No. of days taken for disappearance of diarrhoea		Rise of serum albumin			
									On 10th day		On 30th day	
			Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range
Blend I*	3	3	7.5	5-10	4.3	4-5	3	2-4	1.02	0.9-1.1	2.18	1.65-2.5
Blend II†	5	2-5	10.6	8-15	7.2	6-9	6	4-7	0.91	0.75-1.1	1.89	1.40-2.5

The authorities of the Holdsworth Memorial Mission Hospital, Krishnarajendra Hospital and Government Ayurvedic Hospital provided facilities for this work

*Data from other investigations with skim milk powder and casein*

Skim milk powder	15 <sup>1</sup>	...	6.7	1-12	13.25	7-25	5.2	2-12	0.76	0.20-2.28	1.77	1.03-3.45
	49 <sup>2</sup>	...	12.0	3-33	7.3	0-24	10.5	2-23	0.75	...	1.24	...
	17 <sup>3</sup>	...	18.8	9-34	...	...	14.6	8-24	0.44	...	1.53	...
Casein	2 <sup>4</sup>	2-4	7	6-8	5.8	5-7	3	2-4	0.96	0.8-1.2	1.7	1.5-1.9

<sup>1</sup> School of Tropical Medicine, Calcutta.

<sup>2</sup> Nutrition Research Laboratories, Coonoor.

<sup>3</sup> Medical College, Madras.

<sup>4</sup> Central Food Technological Research Institute, Mysore.

*\*Blend I*

Peanut protein (g)	... 26.0
Soyabean protein (g)	... 33.0
Casein (g)	... 41.0
L-lysine HCl (g)	... 0.75
DL-Methionine (g)	... 1.96

*†Blend II*

Peanut protein (g)	... 52.0
Skim milk powder (g)	... 48.0

The urine was normal excepting for traces of albumin. No parasitic ova, cysts or amoeba were found in the stools in seven of the eight cases. Only one case showed a few round worm ova in the stools. The haemoglobin content was low (30-50 per cent) and red cell count varied between 2.5-3.5 million/cm<sup>3</sup>. Serological tests for syphilis were negative in all the cases. The total protein and albumin contents of serum were very low in all the cases.

## Treatment and Results

All the cases were admitted to the local hospitals. The protein blends were administered to the subjects, the dose being 30 g of protein per child per day. The blends were given in divided doses (6 g protein per dose) five times a day. Each dose was dispersed in 4 to 5 ounces of hot water, sweetened with cane sugar and glucose. The intake of protein on the first two days was maintained at 20 g per day and increased to 30 g per day from the 3rd day onwards. The children also received one ounce of



cane sugar and one ounce of glucose daily. The vitamin supplements were given along with one feed. Vitamin supplements per child per day were as follows:

Vitamin A (I.U.)	... 3,000	Niacin (mg)	... 5.0
„ D (I.U.)	... 400	Pyridoxine (mg)	... 0.5
Thiamine (mg)	... 0.5	Calcium pantothenate (mg)	... 2.5
Riboflavin (mg)	... 0.9		

After the diarrhoea stopped, the children were given daily small quantities of white bread and cooked rice (2 ounces). The subjects, were weighed daily. Blood was drawn on the 10th and 30th day after the beginning of treatment for the estimation of serum proteins, R.B.C. and haemoglobin. The oedema began to show signs of disappearance from 3rd or 4th day and totally disappeared in 6-9 days. The diarrhoea stopped about the 4th-5th day of treatment with protein blends. The dermatosis and hyperpigmentation began to heal from the 10th-12th day of treatment and completely disappeared by about the 30th day. The average loss of weight at the time of disappearance of oedema was about 2 lb and the total gain in weight at the time of discharge (about 18-22 days from the time of reaching minimum weight) was 3-4 lb. The results are given in Table II.

It is proposed to extend the investigations by using various blends of peanut protein and other vegetable protein isolates with or without supplementation with minimal quantities of casein or of l-lysine and dl-methionine.

### Summary

Eight subjects suffering from kwashiorkor were treated with two blends of isolated groundnut and soyabean proteins, casein and skim milk powder. Protein blend I consisted of groundnut protein (26.0 parts), soyabean protein (33.0 parts) and casein (41.0 parts), fortified with lysine (0.75 g) and methionine (1.96g). Protein blend II consisted of isolated groundnut protein (52 parts) and skim milk powder (48 parts).

Each subject received 30g of protein daily from one of the above blends sweetened with cane sugar and glucose. The blends were readily digested by the children and the diarrhoea stopped in about 3-7 days. Oedema disappeared in about 7-10 days on protein blend I and in about 8-15 days on protein blend II.

A marked increase was observed in the serum protein content as a result of treatment. The serum protein content and albumin globulin ratio were brought to the normal after treatment for a period of 30 days with the two protein blends.

### Acknowledgment

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# PROTEIN HYDROLYSATE IN RELATION TO HAEMOPOIESIS AND PROTEIN REGENERATION

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Protein deficiency has been reported in various diseases<sup>1-4</sup> and is now recognised as a frequent complication of medical and surgical diseases<sup>5,6</sup>. The deficiency is characterised by a decrease in the level of haemoglobin in the blood and total protein in plasma, particularly in the albumin fraction. Frequently there is an increase in the globulin fraction.

In order to see how some common ailments affect the state of protein nutrition of the body, and whether any correlation exists between the regeneration of plasma proteins and the synthesis of other proteins in the body, a preliminary study was undertaken in certain pathological conditions. The results are summarised in the present communication.

## Materials and Methods

The cases for study were admitted in the wards with diverse clinical manifestations and were selected for this study on the basis of hypoproteinemia. Thus, there were cases of peptic ulcer, cirrhosis of liver, bacillary and amoebic dysenteries, anaemias of pregnancy, malnutrition, and other oedematous states. On admission, thorough clinical and pathological examinations were done and analyses of plasma proteins, and haematological factors carried out. Cases showing any renal injury were not considered suitable for these studies. Oedema was associated with many of the cases, but no ascites was present. It should be recorded that all the cases of anaemia studied in this series, were of the macrocytic type, though no bone marrow examination could be done to see whether they were all megaloblastic.

After selection, all the cases were put on protein nutrition, both parenteral and oral, for a period of 3-4 weeks or more, and the progress of plasma protein regeneration, and haematological response noted. The preparations used were protein hydrolysates of two types, one prepared from meat protein and meant for intravenous alimentation, and the other prepared from casein and groundnut protein. The former contained 5 per cent hydrolysed protein in the form of amino acids and lower peptides. The oral preparation contained 20 per cent hydrolysed protein.

Before intravenous administration of protein hydrolysate, due precautions regarding any protein sensitivity was taken by carrying out an intradermal wheal test<sup>7</sup>. A record of intake and output was also kept to study the development of any anti-diuretic action after transfusion.

Plasma proteins were fractionated by standard clinical procedures and estimated by nesslerisation after Kjeldahl digestion. The determination of haemoglobin was done according to the method of Sahli-Adams taking 14.5 g of oxy-haemoglobin to be equivalent to 100 per cent haemoglobin.



TABLE I

Clinical condition	No. of cases	Duration of treatment
Peptic ulcer ...	4	30—40 days
Bacillary dysentery ...	4	15—20 „
Amoebiasis ...	3	20—30 „
Anaemias of pregnancy ...	7	20—30 „
Cirrhosis ...	3	40—60 „
Malnutritional oedema ...	2	40—60 „

TABLE II

*Regeneration of plasma proteins, after protein hydrolysate therapy*

Clinical condition	Case No.	Total dose g	Amount of plasma proteins (g) per 100 ml. of plasma					
			Before therapy			After therapy		
			Total protein	Albu-min	Globu-lin	Total protein	Albu-min	Globu-lin
Peptic ulcer	1	...	3.0	2.0	1.0	8.8	5.0	3.5
	2	...	2.8	1.8	1.0	5.4	3.0	2.4
	3	...	3.1	2.2	0.9	5.9	4.0	1.9
	4	...	3.0	1.8	1.2	5.0	3.5	1.5
Chronic bacillary dysentery	1	333	2.8	1.0	1.8	4.4	2.8	1.6
	2	195	5.1	3.0	2.1	5.8	3.6	2.2
	3	88	4.6	2.8	1.8	4.8	3.0	1.8
	4	89	3.8	2.3	1.5	5.2	3.0	2.2
	5	105	4.2	3.0	1.2	4.8	3.6	1.2
Amoebiasis	1	...	4.3	2.5	1.8	4.6	3.5	1.1
	2	...	4.5	2.8	1.7	5.4	4.4	1.0
	3	...	4.5	2.8	1.7	4.8	3.0	1.8
	4	...	4.6	2.9	1.7	6.5	4.1	2.4
Anaemia	1	70	4.2	3.0	1.2	4.3	3.1	1.2
	2	210	4.2	3.0	1.2	4.8	3.3	1.5
	3	270	5.0	3.5	1.5	6.2	4.7	1.5
	4	185	5.0	3.9	1.1	5.4	4.0	1.4
	5	174	4.9	3.0	1.9	5.8	4.3	2.5
	6	233	4.5	3.0	1.5	5.5	3.5	2.0
	7	290	4.5	3.0	1.5	5.8	3.8	2.0
	8	190	4.5	3.2	1.3	5.2	3.4	1.8
	9	200	5.1	3.6	1.5	5.2	3.6	1.6
Cirrhosis of liver	1	...	4.8	1.8	2.9	5.2	2.5	2.7
	2	...	4.5	1.7	2.5	5.0	2.3	2.7
	3	...	4.7	1.9	2.8	5.1	2.5	2.6
Malnutrition with oedema	1	...	4.2	2.9	1.3	5.2	3.2	2.0
	2	...	4.6	1.8	2.8	6.0	3.8	2.0



TABLE III

*Showing the haematological response of cases recorded under Table II after protein hydrolysate therapy*

Haematological response: increase of 0.8 millions = satisfactory; less than 0.8 millions = not satisfactory

Condition	Case No.	Haemoglobin mg		R.B.C. in millions		Haematological response with regard to R.B.C. formation
		Before therapy	After therapy	Before therapy	After therapy	
Peptic ulcer ...	1	4.0	4.8	2.2	3.2	Satisfactory
	2	4.2	9.8	2.1	3.5	"
	3	6.0	12.4	2.5	3.9	"
	4	8.2	10.7	3.0	3.4	Not satisfactory
Chronic bacillary dysentery ...	1	2.9	5.2	2.8	3.7	Satisfactory
	2	2.3	6.9	2.3	3.9	"
	3	6.0	8.2	1.8	2.9	"
	4	8.0	11.3	3.0	4.3	"
	5	8.2	8.9	3.0	3.4	Not satisfactory
Chronic amoebiasis ...	1	7.5	10.8	2.9	4.8	Satisfactory
	2	10.0	12.0	4.2	4.5	"
	3	10.0	12.0	4.0	4.1	Not satisfactory
	4	11.6	14.0	3.8	5.0	Satisfactory
Anaemias ...	1	9.0	10.0	4.0	4.2	Not satisfactory
	2	7.0	8.5	3.35	4.0	Satisfactory
	3	7.0	7.0	2.2	2.4	Not satisfactory
	4	6.5	9.5	1.3	2.2	"
	5	7.0	9.5	2.8	3.0	"
	6	9.0	12.0	2.3	4.0	Satisfactory
	7	1.8	8.0	0.68	2.5	"
	8	6.0	8.5	1.9	3.5	"
	9	7.5	7.6	2.9	3.1	"
Cirrhosis of liver ...	1	8.2	12.5	3.1	4.5	Satisfactory
	2	7.8	11.9	2.93	4.1	"
	3	7.5	12.0	2.65	4.2	"

## Results and Discussion

Though the number of cases are small, the results of the studies bring out some interesting information worth pursuing. It was found that Indian patients, whatever may be the stress of the pathological state, react with an early fall in plasma proteins, and regeneration of the same, even after proper therapy, remains at a sub-normal level for a fairly long time. It is quite possible that all the pathological states, reported herein, have a fairly long chronic history of repetitive relapses, and the low recuperative power of the system may have something to do with such a condition. The rate of regeneration also differs under different clinical conditions, and it is apparent that the increase of plasma proteins does not always correlate with a similar increase of other tissue proteins, as reflected in the amount of haemoglobin formation, or in the total count of erythrocytes in the blood.

Thus, in liver diseases, such as cirrhosis of liver, the rate of regeneration of plasma proteins is extremely low (Table II), though the haematological picture appears more



satisfactory (Table II). It is to be noted that the observation period was the longest in these cases (Table I). With bacillary dysentery cases, however, the protein regeneration proceed fairly rapidly, and can be correlated with a corresponding increase in the haematological picture. It must, however, be agreed that the benefit was the resultant of both protein feeding and proper therapeutic measures. But amoebiasis case do not show such proportional rise in protein synthesis. One remarkable difference between bacillary dysentery and amoebiasis is noticeable with regard to anaemia associated with the conditions. In bacillary dysentery cases, the anaemia is more marked. But amoebiasis cases show only a low degree of anaemia, but a greater stress on protein synthesis, which is possibly associated with the functional disability of the liver usually attending such cases.

The cases of anaemias of pregnancy, however, present a conflicting picture most probably on account of the unknown etiology. Some cases show a satisfactory rise in both plasma protein and haemoglobin formation (cases 6-7), while others record a relatively greater increase of plasma protein with a low haematological response (cases 3 and 9). A third group of cases, show a fully satisfactory increase in haemoglobin but without any significant increase in plasma protein or in erythrocytes (cases 1 and 8).

The diversity of protein synthesis in cases of anaemias, therefore, appears to be related to the diverse and sometimes unknown etiological factors associated in Indian patients. It is remarkable that all the anaemic cases studied were of the macrocytic type, and were treated with suitable haematinics, but still the range of response in these cases presented a wide difference from case to case.

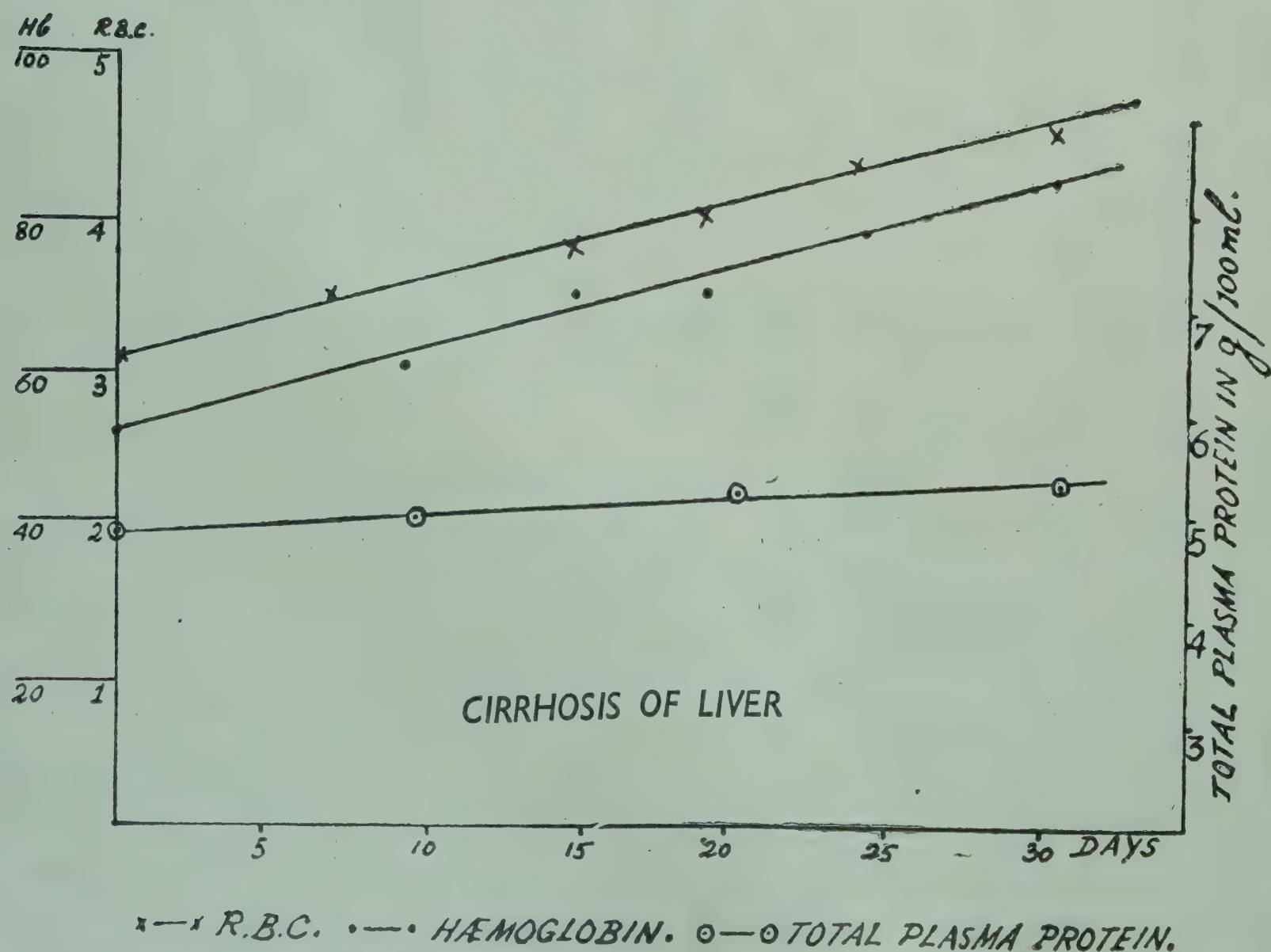


FIG. 1



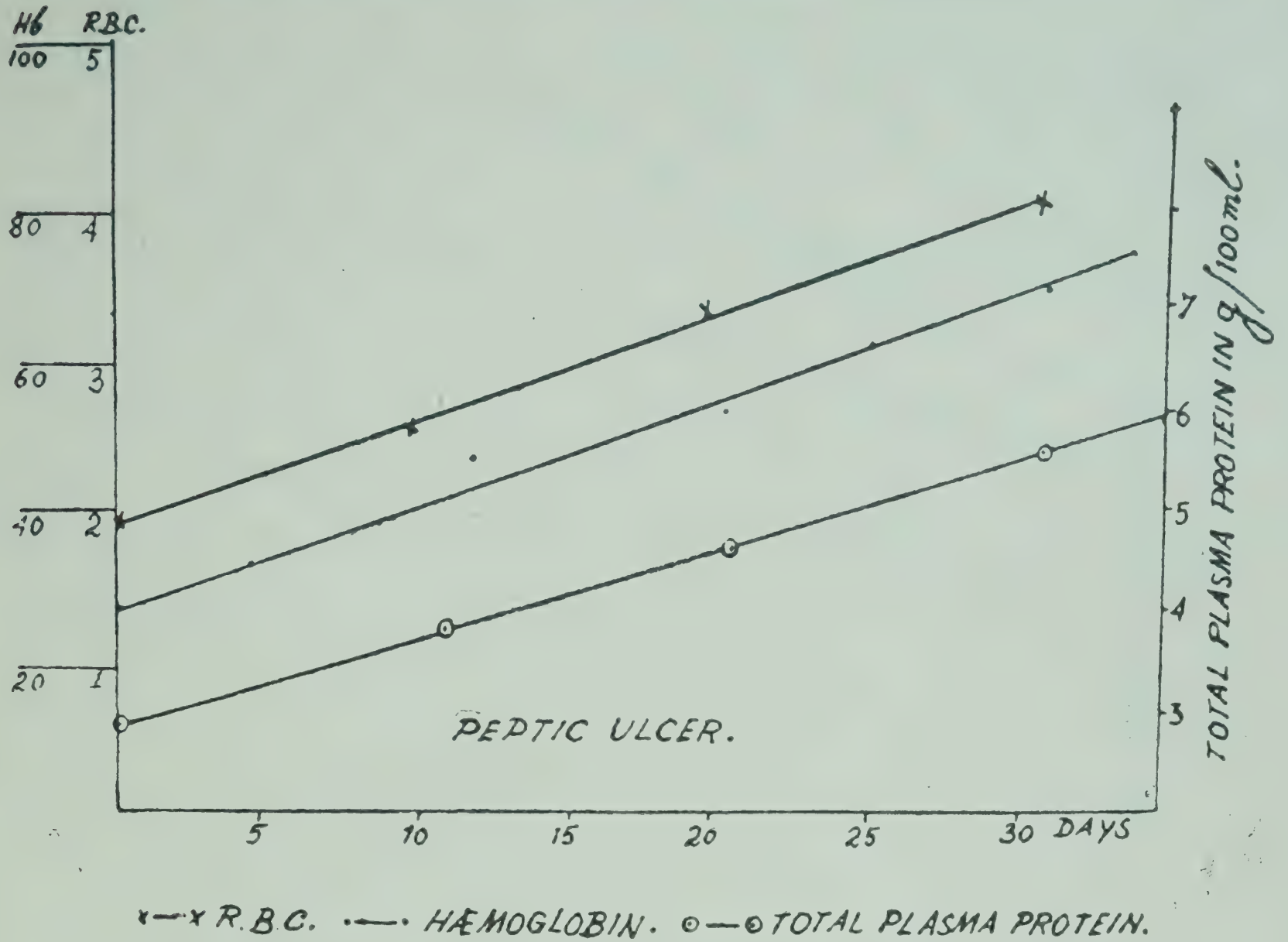


FIG. 2

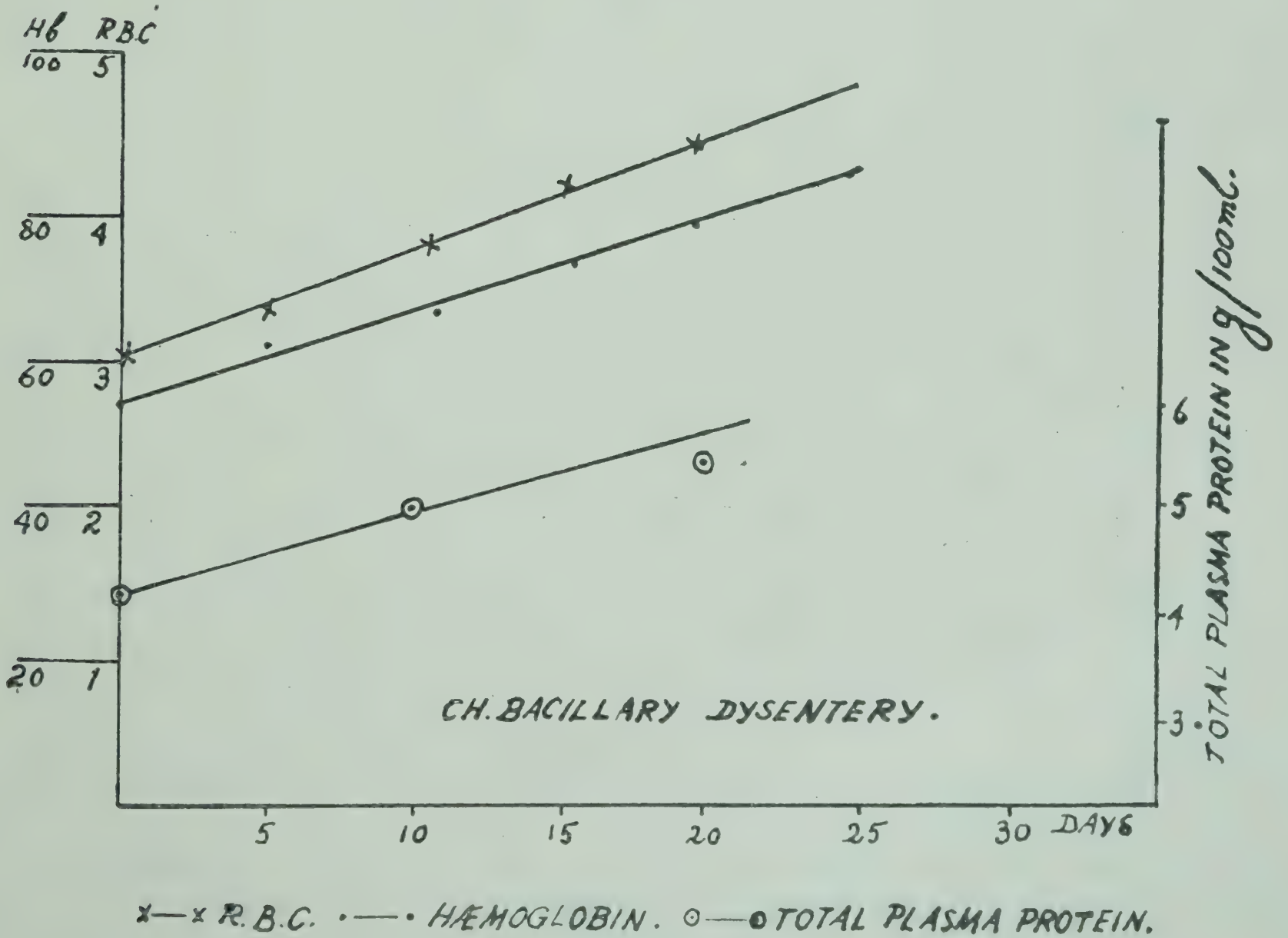
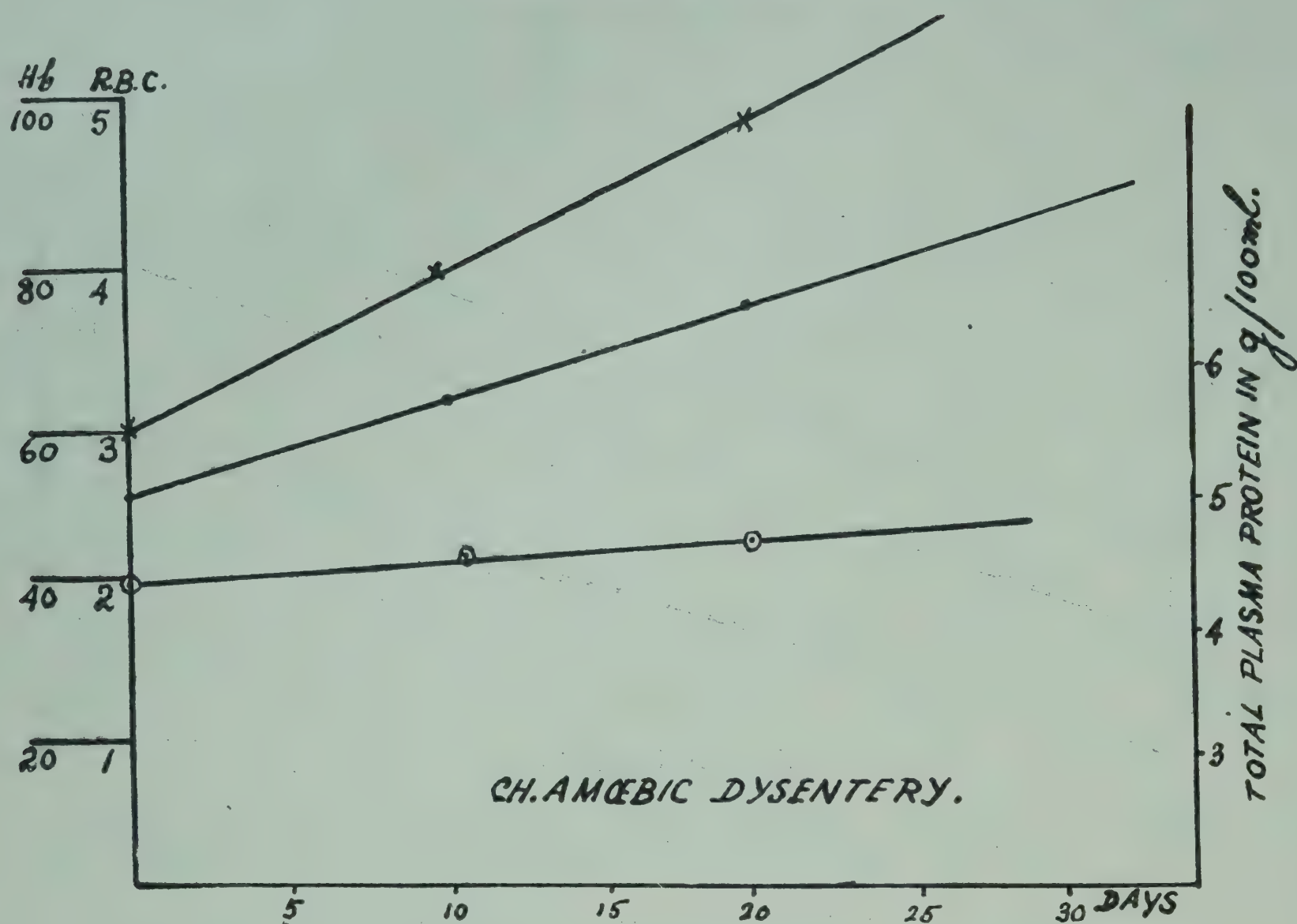


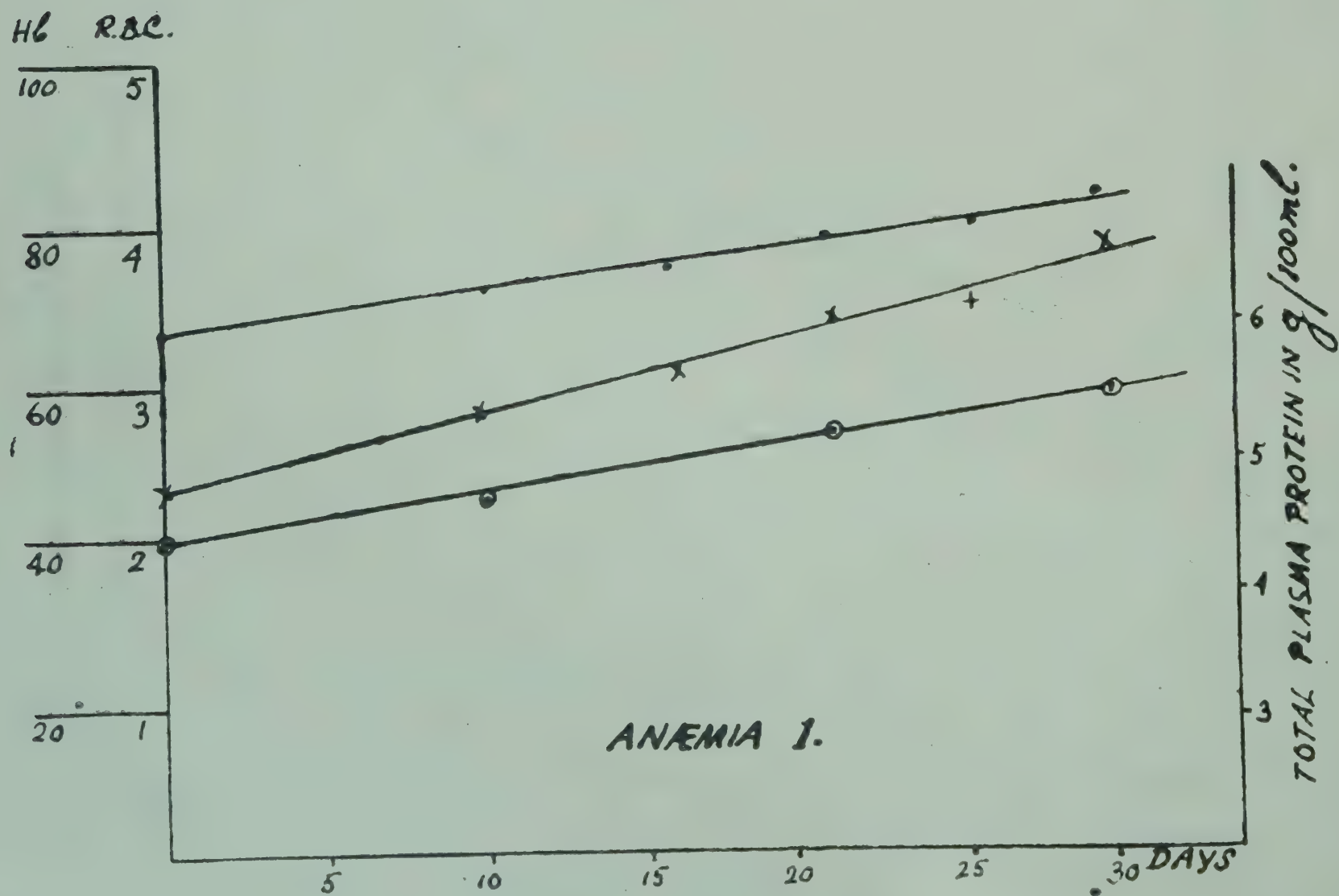
FIG. 3





x—x R.B.C. •—• HÆMOGLOBIN. ○—○ TOTAL PLASMA PROTEIN.

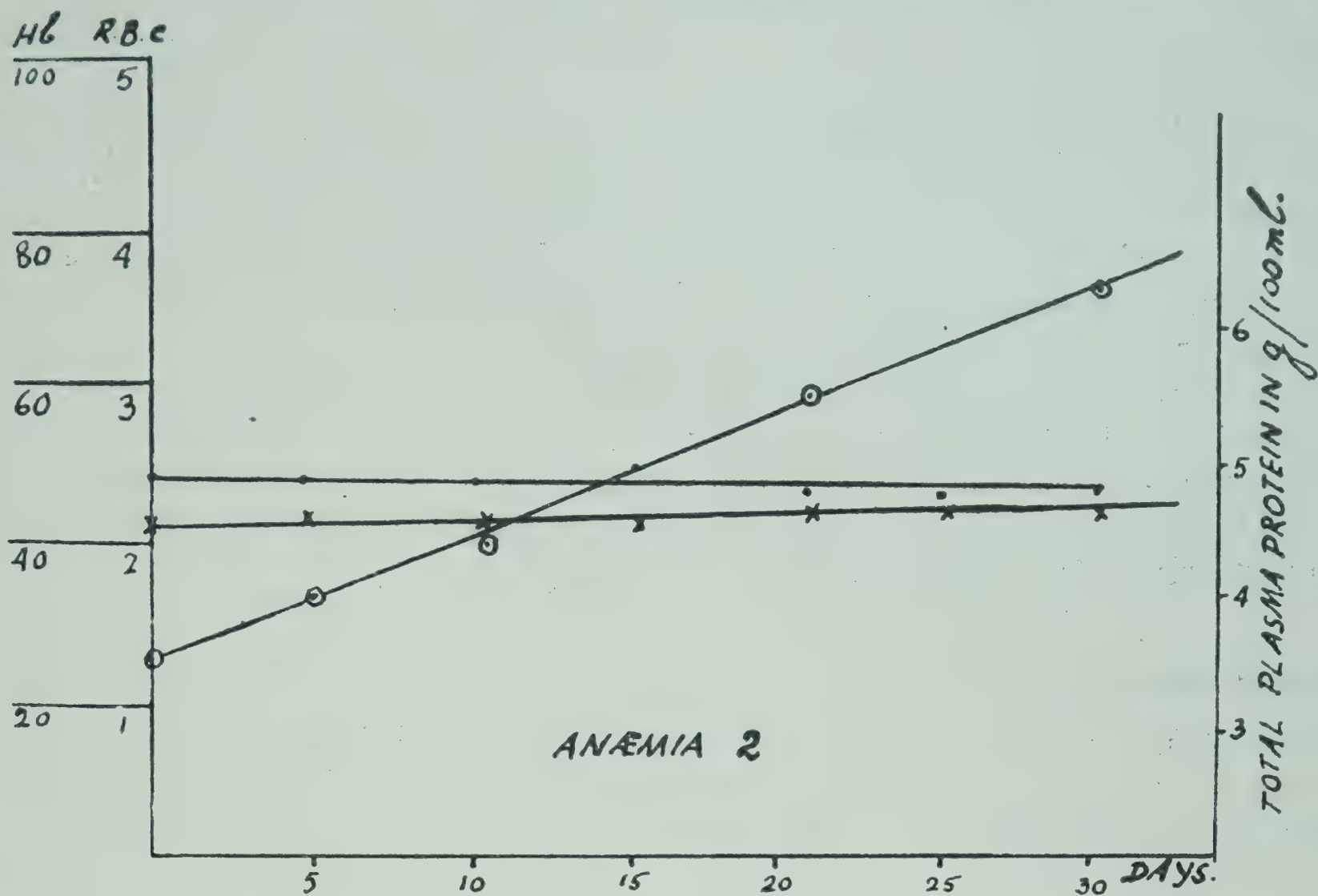
FIG. 4



x—x R.B.C. •—• HÆMOGLOBIN. ○—○ TOTAL PLASMA PROTEIN.

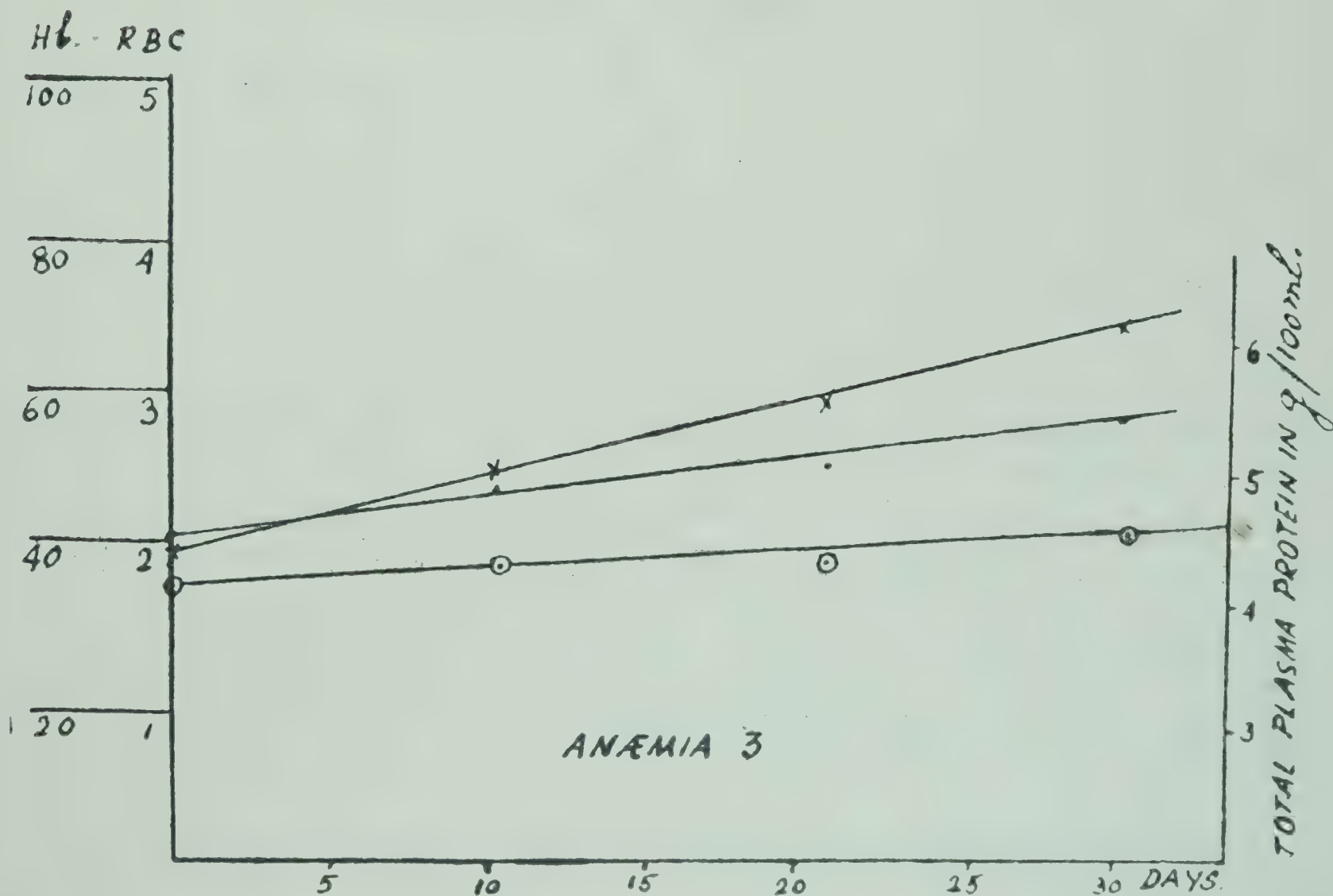
FIG. 5





x—x R.B.C. —•— HÆMOGLOBIN. ○—○ TOTAL PLASMA PROTEIN.

FIG. 6



x—x R.B.C. —•— HÆMOGLOBIN. ○—○ TOTAL PLASMA PROTEIN.

FIG. 7



## Conclusions

From these studies, it is concluded that the synthesis of plasma proteins by liver is more directly affected in liver diseases and in certain types of anaemias. In other clinical conditions not related directly to any possible pathological change in the liver, bone marrow or reticulo-endothelial system, such as in bacillary dysentery and some cases of anaemia, such protein synthesis is probably not directly involved, and due therapeutic measures afford proper correction of the hypoproteinemia.

It is, however, not understood why in spite of all possible therapeutic measures, the rate and level of plasma protein regeneration remains below the expected normal in Indian patients. It is quite possible that all these cases have a fairly long and chronic history of repeated relapses, and the low recuperative power of the system may have something to do with such a condition.

## Acknowledgment

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## Discussion

- Q. What was the source of the protein hydrolysate for intravenous use and its amino acid composition?
- A. The hydrolysates were prepared from meat and fish and contained all the essential amino acids in proper proportion. They contained 5 per cent hydrolysed protein.
- Q. Is the sub-normal rate of regeneration of plasma proteins observed in the patients a racial feature or due to chronic poor liver function or other factors?
- A. The causes have to be carefully investigated.



# BIOCHEMICAL STUDIES IN PROTEIN MALNUTRITION

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Protein malnutrition syndromes constitute some of the major health hazards among the infants and children of our country and continue to draw the concerted attention of clinicians, nutritionists and biochemists to unfold the various facets of its evolution and progress. Recent developments in the concept of diseases have focused the importance of the activities of the enzyme systems in the elaboration and dispensation of chemical substances, which may ultimately be held responsible for the genesis of a diseased state. The reports on the study of various enzyme systems in protein malnutrition syndromes are still meagre in comparison with the large amount of clinical and other laboratory data pertaining to these conditions and even these are mostly devoted to a few selected enzymes from organ preparations like liver, muscles etc.<sup>1,2</sup>.

Since the oxidative reactions in the animal organism are mainly carried out by the enzymes of the Krebs tricarboxylic acid cycle, and as pyruvic and alpha-ketoglutaric acids play pivotal role as intermediaries in the sequential oxidative cycle, it was thought that the level of these acids in the blood and in the urine may portray an overall state of activities of these enzymes in the diseased organism.

## Materials and Methods

*Clinical material:* The clinical material comprised of seven malnourished and three normal children. The age range of the diseased group varied from six months to two years and that of the normal from twelve to eighteen months. All were males. Of the seven malnourished children four had oedema mainly localised on the eyelids and feet. The diseased children were very emaciated, the skin being wrinkled in the axillary and the inguinal folds. Depigmented patches with excoriations on the scrotal and the perioral regions were noticed in two cases and a parchment like skin in two others. The hair was scanty, lustreless and depigmented. In one child, it was red. Body weight varied from 3.1 to 6.4 kg, haemoglobin from 6.5 to 12.5g per cent and red blood cells from 2.2 to 4.7 millions per cu mm. Examination of the stool and urine did not show anything abnormal. In one case, acid fast bacilli (A.F.B.) were found in the gastric washings. The liver was enlarged and palpable in four cases. The relevant data are shown in Table I. X-ray examination did not show any evidence of rickets.

*Collection of samples:* 8-10 ml of blood was collected in the morning from each case from the femoral vein before any feed was given. The blood was immediately transferred to a weighed conical flask containing about 30 ml of 5 per cent (w/v) freshly prepared metaphosphoric acid. The flask was reweighed and the quantity of blood taken for analysis was ascertained. Since the collection of twenty-four hour urine sample in these cases presented certain difficulties, it was decided to restrict the investigation to the morning samples of urine only.



TABLE I

*Showing clinical findings in normal and malnourished infants*

Name	Age in months	Body wt. (in kg)	HB (g%)	R.B.C. (million per cu mm)	Remarks
NORMAL					
B.K. ...	12	10.2	13.6	...	...
S.S. ...	14	12.0	74.5	...	...
B.N. ...	18	14.6	14.0	...	...
MALNOURISHED					
M.D. ...	13	5.1	8.0	3.1	Oedema, liver +
B.S. (2) ...	24	3.5	10.5	3.8	No oedema
B.S. (1) ...	7	4.5	12.5	4.5	No oedema
D.K. ...	22	5.4	6.5	2.4	Oedema, red hair
K.B. ...	6	3.1	9.5	3.6	Oedema, liver + corneal opacity and A.F.B. from gastric washing.
S.K. ...	18	5.7	12.0	4.3	Oedema, liver ++
P.C. ...	8	6.4	8.5	2.7	Oedema, liver ++

Urine samples were collected at the same time by catheterisation into a test tube containing a few drops of concentrated sulphuric acid.

*Method:* A number of methods<sup>3</sup> have been described for the estimation of alpha keto-acids in a variety of biological systems. The method of Taylor and Smith<sup>4</sup> which utilises the principle of preparing nitroquinoxaline derivatives of pyruvic and alpha-ketoglutaric acids in the blood and the urine was adopted.

The standard nitroquinoxaline derivatives of pyruvic and alpha-ketoglutaric acids were prepared by the method of Hockenhull and Floodgate<sup>5</sup> using diaminonitrobenzene (DANB). The DANB derivatives of the standard acids were recrystallised and the melting points of these were found to coincide with those described by Hockenhull and Floodgate<sup>5</sup>.

*Electrophoretic separation of DANB derivatives of pyruvic and alpha-ketoglutaric acids:* The chromatographic separation of DANB derivatives on paper was tried with the solvent system, described by Taylor and Smith<sup>4</sup>, consisting of 5 parts of ethanol, 8 parts of n-pentanol and 6 parts of ammonia (sp. gr., 0.88). The two spots separated after 18 hours and were not very compact. Since paper electrophoretic methods have been employed for separation of dinitrophenyl hydrazine derivatives of alpha-keto-acids<sup>6</sup>, it was deemed worth while to try electrophoretic separation of DANB derivatives of pyruvic and alpha-ketoglutaric acids.



The electrophoretic procedure on paper gave a good resolution of the two DANB derivatives. The horizontal electrophoresis was carried out on a perspex tank using 2.5 cm wide and 34 cm long strips of 3 MM Whatman paper which were mounted on the frame of the electrophoresis apparatus and microlitre quantities of 100 mg per cent DANB solutions of pyruvic and alpha-ketoglutaric acids were applied 11 cm from one end of the paper. The papers were then sprayed with barbitone buffer (pH, 8.6 and ionic strength, 0.025). After a period of one hour to facilitate equilibrium of the buffer solution in the tank and the paper, a constant potential of 150 volts was applied from a standard DC power pack between the two electrodes. The electrophoresis was continued for eight hours in a constant temperature room at 20°C. The paper strips were taken out from the electrophoresis frame and dried in an oven at 60°C for one hour. The DANB derivative of ketoglutaric acid moved 7 cm towards the cathode, while the pyruvic acid derivative moved only 3 cm from the point of application during the eight hour run. The keto-acids were easily seen as yellow spots against the white background of the paper. Further, these DANB spots gave a characteristic brownish fluorescence when seen under an ultraviolet lamp.

*Extraction of DANB derivatives of pyruvic and alpha-ketoglutaric acids from blood and urine:* The preparation and extraction of the DANB derivatives from the samples of blood and urine were done according to the method of Taylor and Smith<sup>4</sup>. Ethylacetate used for the extraction was, however, purified first by treating with 5 per cent sodium carbonate to remove any acid that might have formed and finally by redistillation. The final extracts of DANB derivatives from blood and urine were dried *in vacuo* in small tubes.

The dried extracts were, then, completely transferred to 3 MM Whatman paper using small quantities of acetone. These papers were then taken for electrophoresis. The DANB derivatives of pyruvic and alpha-ketoglutaric acids were identified in the strips by running simultaneously standard solutions of these acids. In some papers, apart from the two identified spots, one and sometimes two other spots were seen between the pyruvic and the alpha-ketoglutaric acid spots from the samples of blood and urine of normal and the diseased group. No attempt was made to identify these spots. Brownish pigmented materials were found at the point of application of DANB extracts from urines in all the strips after electrophoresis. Since these pigmented areas remained distinctly separate from the DANB derivative of pyruvic acid, there was no difficulty in subsequent quantitative assay of the keto-acid derivative.

*Estimation of the keto-acid derivatives:* The areas of pyruvic and alpha-ketoglutaric acid spots were demarcated on the electrophoretograms of samples of blood and urine under an ultraviolet lamp. The areas of paper containing the two DANB derivatives namely that of pyruvic and alpha-ketoglutaric acids were taken in separate test tubes and these were extracted with three changes of 3 ml each of 30 per cent alcohol. The alcoholic extract from each sample, was filtered through a sintered funnel and pooled in a 10 ml volumetric flask and the volume finally made up to 10 ml mark. The optical densities of both the DANB extracts from blood and urine were measured in a Beckman DU spectrophotometer at 780 m $\mu$  and using cuvettes with 10 mm light path. The corresponding paper blanks for pyruvic and ketoglutaric acids were obtained by taking papers from adjacent areas of the electrophoretograms and treating them exactly in the same way. The optical densities of the blanks were also noted.



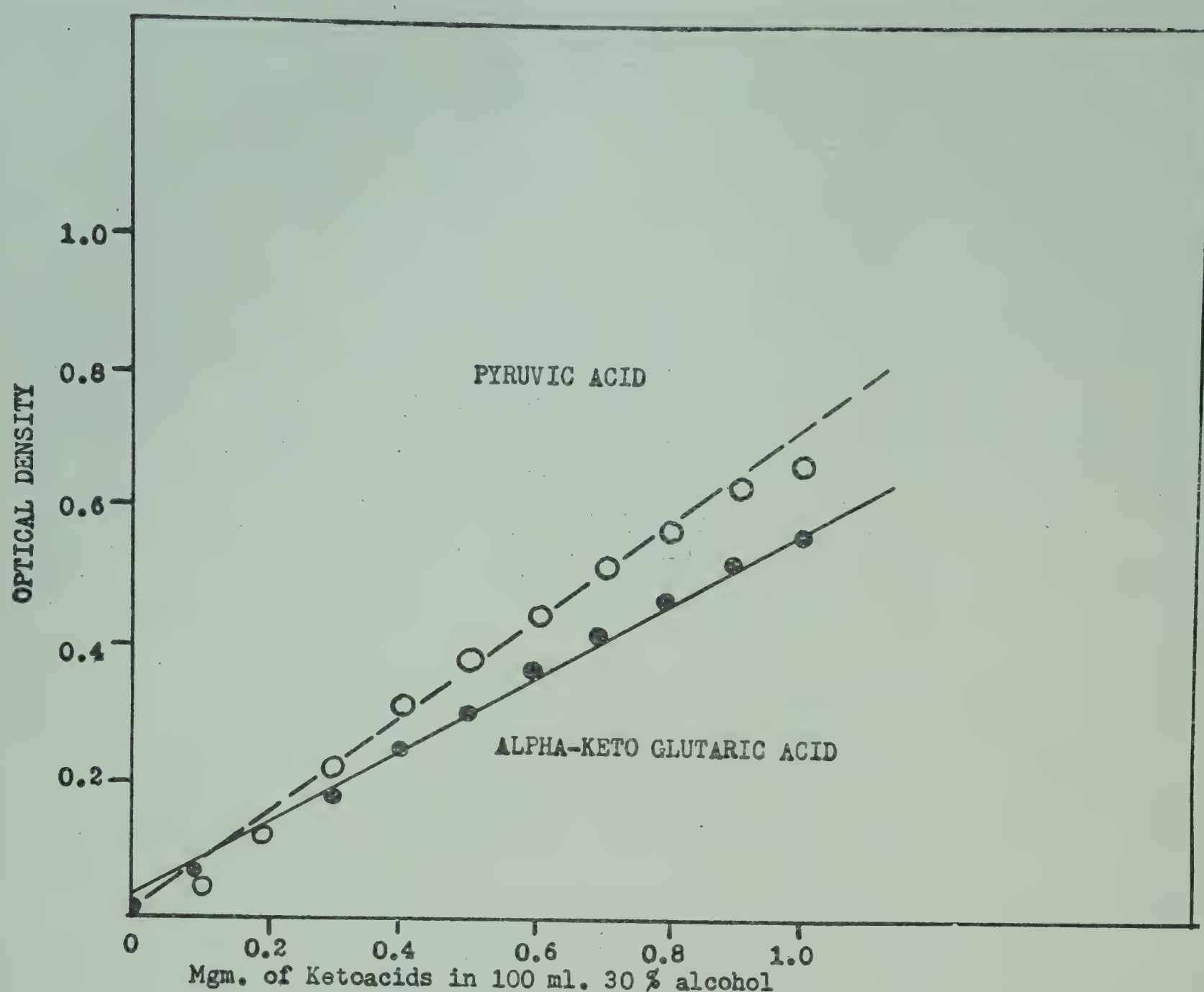


FIG. 1

Calculations were made from the calibration curves constructed from 30 per cent ethanolic solutions of the pure nitroquinoxalinols in various concentrations equivalent to 0.1 to 1.0 mg of keto-acid per 100 ml (the calibration curves are shown in Fig. 1). The accuracy of the method was tested by finding out the percentage recovery of the two DANB derivatives. Three samples each of the two DANB derivatives were taken and treated exactly like that of blood and urine samples and the quantities of the nitroquinoxalinol derivatives were found out after electrophoresis (Table II).

The estimation of the keto-acids in the samples of blood and urine of the patients was carried out at intervals of 11 to 35 days during the course of treatment.

## Results

The values for the keto-acid levels in samples of blood and urine from the diseased and the healthy groups have been given in Tables III and IV.

The data revealed that in all the samples of blood from the malnourished children, there was a rise in the level of pyruvic and alpha-ketoglutaric acids. The rise in the level of pyruvic acid in the blood of malnourished children was less than that of alpha-ketoglutaric acid which was increased two to three folds. Moreover the levels of the keto-acid in the samples of blood were found to decrease during the course of treatment. In patients M.D., D.K., S.K. and P.C., the levels of pyruvic acid in blood fell to the



TABLE II  
Recovery of DANB derivatives of pyruvic and alpha-ketaglutaric acids  
after extraction and electrophoresis

Pyruvic Acid (P.A.) (ug)	Alpha-ketoglutaric Acid (K.A.), (ug)	Found (mg)		Recovery (Per cent)	
		P.A.	K.A.	P.A.	K.A.
40.0	40.0	27.8	34.6	69.5	86.5
40.0	40.0	32.2	36.4	80.5	91.0
20.0	20.0	14.5	17.6	72.6	88.0

TABLE III  
Showing levels of pyruvic and L-ketoglutaric acids in blood and urine of  
dystrophic and normal children

Name		Blood		Urine	
		Pyruvic acid	L-ketoglu- taric acid	Pyruvic acid	L-ketoglu- taric acid
		(mg/100 ml)		(mg/100 ml)	
M.D.	...	0.36	0.40	0.26	0.36
21 days later	...	0.32	0.32	0.22	0.18
35 days later	...	0.21	0.26	0.18	0.20
B.S.	...	0.31	0.31	0.29	0.30
34 days later	...	0.35	0.34	0.16	0.16
35 days later	...	0.33	0.33	0.13	0.14
B.S. 2	...	0.43	0.40	0.13	0.16
31 days later	...	0.28	0.28	0.26	0.32
52 days later	...	0.38	0.41	0.21	0.23
D.K.	...	0.36	0.43	0.24	0.22
12 days later	...	0.28	0.28	0.26	0.32
19 days later	...	0.28	0.26	0.30	0.34
32 days later	...	0.26	0.18	0.13	0.17
K.B.	...	0.34	0.40	0.16	0.37
21 days later	...	0.36	0.44	0.45	0.51
43 days later	...	0.40	0.33	0.30	0.42
S.K.	...	0.36	0.27	0.26	0.23
15 days later	...	0.33	0.27	0.18	0.15
26 days later	...	0.20	0.19	0.37	0.52
P.C.	...	0.34	0.29	0.26	0.23
15 days later	...	0.28	0.19	0.42	0.49
		Findings in normal children			
B.K.	...	0.25	0.15	0.41	0.44
S.S.	...	0.28	0.17	0.43	0.57
B.N.	...	0.24	0.12	0.43	0.60



TABLE IV  
*Pyruvic and  $\alpha$ -ketoglutaric acid levels in blood and urine of normal and dystrophic children before commencement of treatment*

		Pyruvic Acid		Alpha-ketoglutaric acid	
		Normal	Dystrophic	Normal	Dystrophic
Blood	...	0.24—0.28	0.31—0.43	0.12—0.17	0.27—0.40
(mg/100g)	...	(0.26)	(0.36)	(0.14)	(0.33)
Urine	...	0.41—0.43	0.13—0.29	0.44—0.60	0.16—0.31
(mg/100 ml)	...	(0.42)	(0.23)	(0.53)	(0.30)

Values within brackets are the averages.

normal limit during the period of treatment. The level of alpha-ketoglutaric acid in these patients, though showing a fall, was still higher than those of the control series. All of them showed significant clinical improvement during this period. Rest of the patients B.S. (1), B.S. (2), and K.B. did not show any significant alteration in the levels of both the keto-acids in blood during the period of treatment, and did not show any clinical improvement; only in K.B. the oedema disappeared. Acid fast bacilli were demonstrated in the gastric washings of this patient.

In the malnourished group, the level of pyruvic and alpha-ketoglutaric acids in the urine varied from 0.13 to 0.29 mg and 0.16 to 0.37 mg per 100 ml. In the control series, however, the level of both the keto-acids was higher in urine, that for pyruvic acid and alpha-ketoglutaric acid being 0.41 to 0.43 mg and 0.44 to 0.60 mg per 100 ml respectively.

In a preliminary study total proteins and lipoproteins in sera were estimated in six cases of protein malnutrition and six normal cases. Table V. The serum protein was estimated by Markhams' modification of the micro-Kjeldahl method. The lipoproteins were separated by paper electrophoresis using veronal-acetate buffer of pH 8.6, ionic strength, 0.05 and a potential gradient of 6 volt/cm. The separation into alpha, beta and 0 fractions were complete between 4 and 5 hours. The electrophoretic technique employed was similar to that describe by McDonald and Bermes<sup>10</sup>. The sera were prestained with acetylated Sudan Black B. The acetylation of the dye was carried out following the procedure of Lillie and Burtner<sup>11</sup>.

The studies on lipoproteins in protein malnutrition have not been reported earlier except for a casual report on a single case by Salt and Wolff<sup>12</sup> when they described the lipoprotein changes in sera in different diseases in children. The most remarkable finding in our studies was a significant fall in the  $\alpha$ -fraction. In two cases,  $\alpha$ -lipoprotein band was absent in electrophoretogram (Fig. 3). The  $\beta$ -band was found to be higher and was found to have moved further than the  $\beta$ -band of the normal. There was, thus, a better separation between the 0 and  $\beta$ -band in the diseased group. Further the  $\beta$ -band was wider than that of the normal.



TABLE V

*Total protein and lipoprotein fractions in normal and malnourished children*

Name	Age in months	Body wt (in kg)	Total Protein (g%)	$\alpha$ %	$\beta$ %	$\gamma$ %	Ratio ( $\beta/\alpha$ )
NORMAL							
B.R.	12	9.0	5.05	18.7	28.3	53.0	1.50
M.D.	24	10.0	7.15	21.1	25.6	53.3	1.20
S.K.L.	18	10.0	6.80	22.3	22.8	54.9	1.10
P.G.	24	11.0	6.60	18.1	18.8	63.1	1.04
D.B.	36	15.0	6.80	26.7	28.5	44.8	1.07
D.B.	42	18.0	6.50	21.0	41.1	37.9	1.90
MALNOURISHED							
P.P.	24	6.0	6.5	1.9	31.4	66.7	31.40
S.P.	24	6.5	5.5	Nil	84.3	15.7	$\alpha$
K.	18	6.0	6.0	Nil	63.8	36.2	$\alpha$
H.M.	36	7.5	5.0	5.1	34.4	60.5	6.70
D.R.	24	4.0	5.05	3.0	54.8	42.2	18.30
S.B.	24	4.0	5.80	6.7	39.1	44.2	5.80

## Discussion

The increase in the levels of pyruvic and alpha-ketoglutaric acids in the blood of the malnourished children indicate a derangement of the enzyme systems concerned in metabolism of these substances. Since both the keto-acids are intermediaries in the Krebs cycle, it is reasonable to expect damage to the enzymes of the Krebs cycle. Recent studies with radioactive C14 pyruvate demonstrate that in rats pyruvate is metabolised via oxalacetate to a greater extent in the liver than elsewhere in the carcass<sup>14</sup>. As evidence exists for damage to the liver<sup>7,1</sup> in protein malnutrition syndrome of children, the rise in pyruvic acid level may be accounted for by failure of the liver enzymes to convert pyruvic acid to oxalacetate and thus damp the sequential oxidative steps of the Krebs cycle.

The increase in the alpha-ketoglutaric acid level in the blood is comparatively much higher than that of pyruvic acid in malnourished children. It is unwise to speculate on the metabolic derangements in malnutrition syndrome leading to an excessive increase in the level of the alpha-ketoglutaric acid, since much less is known about the metabolism of this particular keto-acid in the mammals. It occupies the key position in the branching points for diverse metabolic pathways like transamination, oxidative decarboxylation etc. A definite explanation for the higher rise in ketoglutaric acid level compared to that of



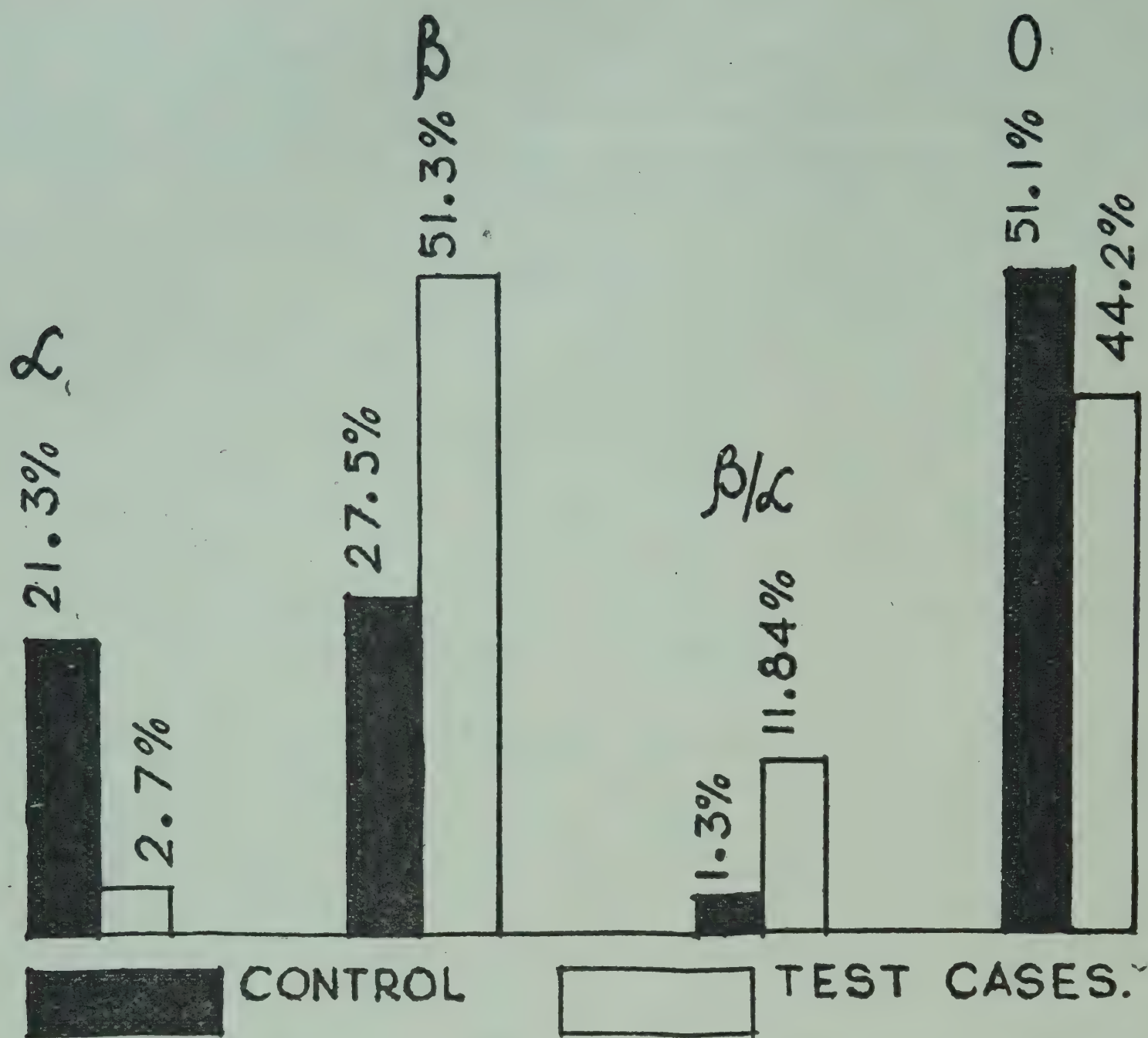


FIG. 2

pyruvic acid in blood in malnutrition, can be offered only when all the details of the various enzymes responsible for its handling have been worked out. It is known that alpha-ketoglutaric acid needs a complex mechanism involving at least six co-factors, namely coenzyme A, lipoic acid, co-carboxylase, adenosine diphosphate, guanosine diphosphate and inorganic phosphate to shunt it into the Krebs cycle<sup>3</sup>. Moreover the size and distribution of the metabolic pool of alpha-ketoglutaric acid has not been studied yet. Hence availability of any of the co-factors either alone or in combination might be responsible for defective handling of the keto-acids by the enzymes of Krebs cycle. In addition simultaneous derangements of other enzyme systems metabolising alpha-keto-acids may account for the increased of keto-acids in blood.

The levels of the keto-acids in the samples of urine were found to be much higher than the corresponding values for the blood. Further, it was found that the quantity of both the keto-acids were higher in the urine of the normal group than that of the malnourished group. It is possible that the levels of keto-acids in the fasting samples of urine do not portray the overall excretion pattern of these acids. However, recently, evidence has been provided to indicate active participation by the kidneys in the metabolism of pyruvic and ketoglutaric acids in human subjects<sup>8</sup>. It has also been demonstrated that the patients suffering from malnutrition syndrome excrete large quantities of amino acids in urine and the aminoaciduria is of renal origin<sup>9</sup>. The altered levels of ketoacids in the urine of the



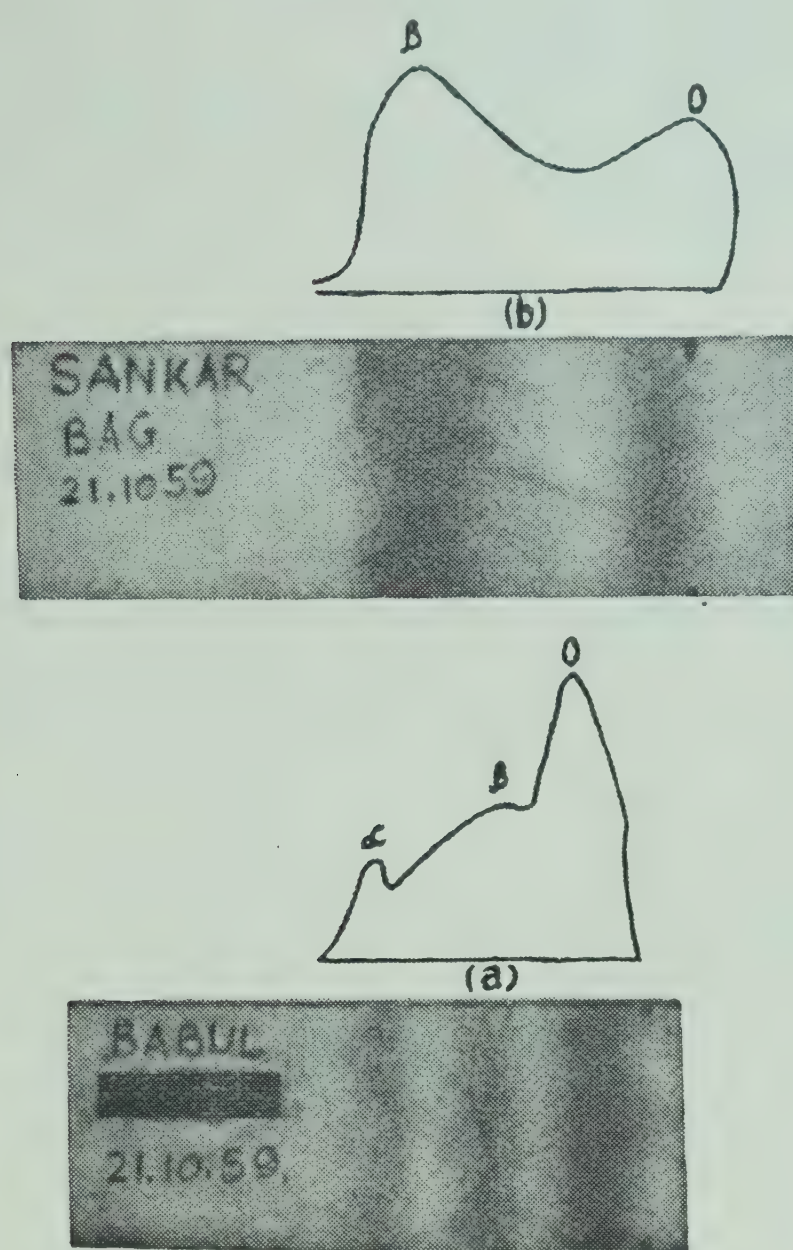


FIG. 3. Electrophoretogram of serum lipoprotein

- (a) In a normal child  
 (b) In a case of protein malnutrition syndrome

malnourished group may, therefore, arise as a result of derangement in the functional activities of the kidneys, and the levels of keto-acids in blood may be partly a reflection of the state of activities of the kidney. The interpretation of the findings regarding the serum total protein and lipoprotein levels in normal and malnourished children has to await further work now in progress.

### Summary

1. Pyruvic acid and alpha-ketoglutaric acid were estimated in blood and urine of seven malnourished and three normal children.
2. A new electrophoretic method of separation of DANB derivatives of pyruvic and alpha-ketoglutaric acid has been described.
3. The pyruvic and alpha-ketoglutaric acid levels in blood of malnourished children were raised when compared to that of normals.
4. The urinary excretion of pyruvic and alpha-ketoglutaric acids were found to be less in the morning samples of the malnourished group.



5. The findings have been discussed in relation to the utilisation of the keto-acids by the enzymes of the Krebs cycle.
6. The lipoproteins of the sera have been separated by electrophoresis and the findings in the control and the test cases are recorded.

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# INDIAN MULTIPURPOSE FOOD—A LOW COST PROTEIN SUPPLEMENT TO THE INDIAN DIETARY

RADHA KARNAD

It is now generally recognised that the diets consumed by a large majority of the population, especially of the low income groups, in India are deficient in various important constituents such as proteins, vitamins and minerals. The consequences of these deficiencies are strikingly seen in the case of the vulnerable segments of the population. Nutritional surveys carried out in the country have shown that protein malnutrition (Kwashiorkor), and diseases due to deficiencies of vitamin A and B-complex and iron are widely prevalent, especially among growing children and expectant and nursing mothers. Protective and protein rich foods like milk, meat, egg, etc., are not available in sufficient quantities in the country and further, are not within the reach of the low income groups. There is therefore an urgent need for the large scale production of low cost protein supplements from available protein-rich raw materials and fortifying them with vitamins and minerals. These protein-rich foods can be used as a supplement to the poor dietaries and also for the treatment and prevention of protein malnutrition.

The protein-rich foods available in abundance in the country are the various edible oilseed meals and pulses. Subrahmanyan *et al*<sup>1</sup> have developed a low cost protein food, known as Indian Multipurpose Food (MPF) which consists of a blend of 75 parts of low fat groundnut flour and 25 parts of Bengal gram (*Cicer arietinum*) flour fortified with vitamins and minerals. Extensive investigations<sup>2, 3</sup> have shown that Indian Multipurpose Food possesses high nutritive value and can be used as an effective supplement to the poor dietaries based mainly on cereals.

The Meals for Millions Association of India has been distributing and popularising the Indian Multipurpose Food. A number of feeding trials at different centres in the country were initiated with the co-operation of the Central Food Technological Research Institute, Mysore, to evaluate the supplementary value of the food to the diets of children. The results of these studies are presented in this paper.

## Feeding trials with Indian Multipurpose Food

Subrahmanyan *et al*.<sup>3</sup> have shown that a supplement of 2 oz of Indian MPF for a period of 5 months to diets of undernourished children brought about a significant improvement in their growth and nutritional status. Similar studies were continued in several centres (nearly 300 institutions in different parts of the country), the period of the experiments ranging from 3 months to 1 year. The institutions were mostly schools, students' hostels, orphanages, children's homes, T.B. sanatoria and pediatric wards in hospitals. The results of some of these studies are briefly described below:

*Trials at Ankola:* Feeding trials at Ankola were carried out in collaboration with CFTRI Children aged 2-6 years (both girls and boys) were selected and divided into two groups on the basis of their initial height and weight. One group received their usual



diet, serving as the control and the other received a daily 1 oz supplement of Indian MPF. 1 oz of the supplement supplied the following nutrients.

TABLE I  
*Nutrients supplied by 1 oz of Indian Multipurpose Food*

Nutrients		Content per oz
Protein (g)	...	12.0
Calcium (mg)	...	190
Phosphorus (mg)	...	234
Iron (mg)	...	1.5
Vitamin A (I.U.)	...	857
Thiamine (mg)	...	0.4
Riboflavin (mg)	...	0.4
Nicotinic acid (mg)	...	4.0
Vitamin D (mg)	...	71

The feeding was continued for a period of 6 months. Measurements of height, weight, RBC count and haemoglobin content were made both at the beginning and at the end of the experiment. The children were also assessed for their nutritional status. The results are given in Tables II and III. From the results it is clear that the children receiving the supplement of Indian MPF improved significantly in their nutritional status and showed improvements in height, weight, RBC count and haemoglobin contents over the control.

TABLE II  
*Results of the feeding experiments with Indian Multipurpose Food (1 oz of MPF) as a supplement per day on the growth and nutritional status of children at Ankola*

	Boys between the ages of 2-6 years						Boys between the ages of 7-12 years					
	Control group			Experimental group			Control group			Experimental group		
	Initial	Final	Diff.	Initial	Final	Diff.	Initial	Final	Diff.	Initial	Final	Diff.
Height in inches ...	36.3	37.6	1.3	36.09	37.61	1.52	48.57	49.78	1.21	46.53	48.08	1.55
Weight in pounds ...	27.26	28.44	1.18	26.88	29.60	2.72	65.00	46.80	1.80	43.00	47.69	4.68
Haemoglobin gram %	8.64	9.30	0.66	8.98	11.09	2.11	9.43	10.47	1.04	9.00	11.32	2.32
Total nurtitional score	3.00	2.60	-0.40	3.8	1.4	-2.4	2.8	2.4	-0.4	2.6	1.0	-1.6
<i>Changes in the nutri-tion status</i>												
Inproved ...	...	8	...	...	21	...	...	2	...	...	11	...
Stationary ...	...	36	...	...	20	...	...	20	...	...	6	...
Deteriorated ...	...	4	...	...	...	...	...	...	...	...	...	...
2-6 years of age						7-12 years of age						
Total No. of boys in the control group						...						
do experimental group						...						
Duration of the experiment						6 Months						

*Trials at Calcutta:* Feeding trials were carried out in an orphanage with 43 boys and 48 girls under the guidance of the Medical Officer-in-charge. The plan of the experiment was the same as described above. Children below 12 years received daily a supplement of



TABLE III  
*Results of the feeding experiments with Indian Multipurpose Food (1 oz of MPF) as a supplement per day on the growth and nutritional status of children at Ankola*

	Girls between the ages of 2-6 years						Girls between the ages of 7-12 years					
	Control group			Experimental group			Control group			Experimental group		
	Initial	Final	Diff.	Initial	Final	Diff.	Initial	Final	Diff.	Initial	Final	Diff.
Height in inches	36.60	38.20	1.60	35.80	37.71	1.91	49.82	51.23	1.41	47.98	49.58	1.60
Weight in pounds	27.43	28.92	1.49	26.11	29.37	3.26	47.60	48.79	1.19	45.31	49.98	4.67
Haemoglobin in grams% ...	8.67	9.45	0.78	8.76	11.30	2.54	9.61	9.87	0.26	9.34	11.95	2.61
Total Nutritional score ...	2.60	1.80	-0.80	2.80	1.20	-1.60	2.60	2.40	-0.20	3.70	1.50	-2.20
<i>Changes in the nutritional status</i>												
Improved ...	...	10	...	...	39	...	...	...	...	...	20	...
Stationery ...	...	29	...	...	24	...	...	14	...	...	8	...
Deteriorated ...	...	1	...	...	...	...	...	2	...	...	...	...

	2-6 years of age	7-12 years of age
Total No. of girls in control group	... 40	16
do experimental group	... 63	28
Duration of the experiment	6 Months	

$\frac{1}{2}$  oz and those above 12 years, 1 oz. The supplement was given along with their afternoon tiffin consisting of beaten, and fried rice, milk, suji, etc. Nutro biscuits ( $\frac{1}{2}$  oz) were also given in addition to the MPF supplement. Feeding was continued for a period of 1½ months. Periodical medical check up and records of heights and weights of children under study were regularly done by the Medical Officer. From this report it could be concluded that even during the short period of feeding trials with MPF and Nutro biscuits, the results obtained were very encouraging. It was also observed that children, not receiving the supplements deteriorated in their nutritional status.

*Trials at J. J. Hospital and Grant Medical Hospital, Bombay:* Trials carried out with children receiving a supplement of Nutro biscuits showed that the biscuits were quite acceptable, caused no digestive troubles and that there was a definite improvement in the health of the children receiving the supplement. Most of the children gained 1-2 pounds in a period of 3-4 weeks when given the supplement over and above the hospital diet.

Several reports of feeding trials have been received from different centres, where MPF was given in the form of porridge with skim milk powder. All the reports have confirmed the improvements in height, weight and nutritional status of children.

*Use of MPF in school lunch programmes and homes:* Indian MPF is versatile as it can be easily incorporated in a large number of common food preparations without



affecting the taste, at the same time enhancing the nutritive value. With the co-operation of house wives, MPF has been tried in several homes. One of the popular preparations was found to be biscuits made by using MPF. The author's experience has further shown that MPF can be easily introduced in school lunch programmes as porridge with skim milk powder. Other snacks which are equally acceptable are the biscuits, barphi and chikki containing MPF.

### Nutro-biscuits in diet therapy in cases of nutritional oedema syndrome

Several workers have successfully treated cases of nutritional oedema syndrome (Kwashiorkor) by oral administration of various types of protein supplements. Mention may be made here of soyabean and ripe banana used by Dean<sup>4</sup>, cooked Bengal gram flour by Venkatachalam *et al.*<sup>5</sup> and Indian MPF formula C (containing skim milk powder) by Subrahmanyam *et al.*<sup>6</sup>.

Investigations were carried out using Nutro biscuits in the diets of children suffering from protein malnutrition. 1 oz of the Nutro biscuits supplied the following nutrients.

TABLE IV

*Nutrients supplied by 1 oz of Nutro biscuits*

<i>Nutrients</i>	<i>Nutro biscuits</i> (Values per oz)
Protein (g)	10.3
Calcium (mg)	140
Thiamine (mg)	0.4
Riboflavin (mg)	0.85
Nicotinic acid (mg)	3.95
Vitamin A (I.U.)	852
„ D (I.U.)	85
Calories	105

82 children suffering from malnutrition in the pediatric wards of Government Stanley Hospital, Madras were given nutro biscuits. The children were between the age groups of 6 months—1½ years; 1½—3 years; and 4—11 years. The feeding was continued for a period of 6 weeks. From the results the following conclusions were drawn.

Nutro biscuits were readily accepted by children below 3 years and were found to be ideal for older children (over 3 years) and therefore, might be used as a prophylaxis against protein malnutrition. The biscuits were easily digested and no adverse effects were noticed.

Detailed reports of two cases of protein malnutrition treated with nutro biscuits are given below. Two children (aged 5-5½ years, were admitted to the hospital with oedema, skin pigmentation, anorexia and diarrhoea). One of the subjects showed enlarged liver and sparse reddish hair. The children were given a diet which provided 40 calories



and 4 g of protein per kilo of body weight per day. The protein in the diet was derived mainly from Nutro biscuits.

The oedema began to diminish and gradually subsided in about 5 weeks. The skin lesions healed in about 7-8 weeks and the children improved in their general condition and put on weight. The serum protein was estimated both at the beginning and at end of the treatment and the results are given in Table V.

TABLE V  
*Serum protein content of subjects before and after  
treatment with Nutro biscuits*

Serum proteins	Subjects			
	I		II	
	Initial	Final	Initial	Final
Total protein (%)	3.24	5.48	4.28	6.56
Albumin (%)	1.00	3.20	2.28	4.28
Globulin (%)	2.24	2.28	2.00	2.28

Normally cases of protein malnutrition in the hospital were treated with skim milk powder and these cases were available for comparison and it was found that both as regards final results as well as time required for improvement, nutro biscuits compared favourably with skim milk powder.

## Summary

Feeding trials carried out at different centres have shown that Indian Multipurpose Food (1 oz) and Nutro biscuits (1 oz or 1/2 oz) when supplemented daily to the diets of undernourished children produced a marked improvement in their growth and nutritional status.

Consumer trials carried out in several homes and educational institutions and hospitals have shown that Indian Multipurpose Food can be incorporated in common food preparations.

Clinical trials with Nutro biscuits have been carried out at the Government Stanley Hospital, Madras, in the treatment of cases of nutritional dystrophy in children. The results indicated that Nutro biscuits are acceptable to most of the children and they do not develop any symptoms of indigestion. Two children suffering from oedema all over the body, anorexia and pigmentation of the skin were successfully treated by giving a supplement of Nutro biscuits as the sole source of protein. Oedema diminished soon after the treatment and completely subsided in about 5 weeks. The general condition of the children improved, the skin lesions gradually healed and disappeared after 7-8 weeks.



## Acknowledgments

My thanks are due to Dr V. Subrahmanyam, Director, Central Food Technological Research, Mysore, Institute for permission to include results of feeding trials carried out at Ankola.

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# THE PLACE OF A COMPOSITE PROTEIN FOOD IN SCHOOL LUNCH PROGRAMMES

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One of the chief deficiencies of the diets of school children in India is protein which is essential during the growing period. Various groups of workers in India have shown the importance of supplementing the diet of school children with protein rich foods. Aykroyd and Krishnan<sup>1</sup> and Lal and Bose<sup>2</sup> have demonstrated the supplementary effect of skimmed milk powder. Vegetable milk curd prepared from seeds such as soyabean and groundnut has been shown to supplement the diet of school children<sup>3, 4</sup>. The supplementary effect of multipurpose food and tapioca macaroni has been reported by different groups of workers<sup>5, 6, 7, 8</sup>. It has also been demonstrated that composite protein foods such as a blend of coconut meal, low fat groundnut flour and Bengal gram flour are effective supplements to the diets of school children<sup>9, 10, 11</sup>. The importance of school lunch programmes has been realised by the Governments of different States as well as various organizations in India<sup>12, 13</sup>.

The work presented in this paper focuses attention on the importance of supplementing school lunches with cheap protective foods in the form of snacks. Groundnut (*Arachis hypogea*) Bengal gram dhal (*Cicer arietinum*) and skimmed milk powder were selected as the ingredients of the composite protein food because of the high protein content and low cost.

## Experimental Procedure

*Choice of volunteers:* 'Bala Mandir', an orphanage which is the Madras state branch of the Indian Council of Child welfare for destitute children was selected for the investigation. It was chosen because the food given to every child is controlled, the children do not get any extra food other than their usual diet, they stay in the orphanage throughout the year and large number of children of the same age group were available.

42 children between the ages of 7 and 9 were chosen. On the basis of their age, the children were paired and the members of each pair were allotted at random to two groups of 21 each. The duration of the experiment was ten weeks.

*Diet consumed by the children under study:* The composition and the calculated nutritive value of the orphanage diet are given in Tables I and II. Samples of food given to the children were collected on three alternate days in a week and analysed for protein by the micro Kjeldahl method<sup>14</sup>. The analysis was done during the first and sixth week of experiment. A second analysis was done because rice, wheat or ragi was introduced into the diet in place of American corn at the beginning of the fourth week of the experiment. The protein content of the diet varied from 44.7 g to 52.4 g at the beginning of the experiment and from 40.2 g to 42 g after the change in the diet. Samples of lunch given to the age groups of seven and nine were also collected







TABLE III  
*Food value of the supplements*  
 (Calculated according to *Hlth Bull.*, No. 23, 1956)

Nutrient		Protein supplement	Iso-caloric supplement
Calories	...	366	367
Carbohydrate (g)	...	36.6	64.1
Protein (g)	...	26.0	0.3
Fat (g)	...	12.9	12.2
Calcium (mg)	...	424.0	32.0
Phosphorus (mg)	...	488.0	15.5
Iron (mg)	...	3.0	3.6
Vitamin A (I.U.)	...	...	7.9
Thiamine (I.U.)	...	16.0	10.5
Riboflavin ( $\mu$ g)	...	11.0	...

The supplement was given as the first item of their lunch for both groups. The experimental group ate their supplement mixed with the sambar rice. The control group took the sambar rice after the payasam. Both groups finished their lunch with curds rice.

*Collection of data:* Heights and weights of the children were taken once a week. The nutritional deficiency score, using the score card framed by the Indian Council of Medical Research (Nutrition Advisory Committee, 1948) was recorded at the beginning and at the close of the experiment by the same observer. Hip width measurements were taken at the same time as the nutritional deficiency score. Red blood cell counts were determined once a fortnight. Haemoglobin levels were obtained every fortnight beginning after the 12th day of experiment. It was not possible to estimate the initial haemoglobin level as the children were nervous and sufficient blood could not be drawn. Blood proteins (albumen, globulin) were estimated on samples taken from the median basilic vein.

Red blood cell count was done by the standard micropipette method<sup>15</sup>. Acid haematin method was used for haemoglobin estimation. Blood proteins were estimated by the Microkjeldahl method<sup>16</sup> and by the Koch and McMeekin method<sup>14</sup>.

## Results and Discussion

The results are presented in Table IV and Figures 1 to 4.

*General appearance:* Increased vigour and alertness were observed in the children of both groups. Although these do not lend themselves to measurement the objective impressions consistently recorded are a good evidence of nutritional improvement. The improvement in the control group may be explained as being due to psychological reasons such as individual attention at meal times and when the measurements were taken.

All the children who showed angular stomatitis in the experimental group at the start of the experiment were free from it by the fourth week of experiment with no recurrence during the experimental period. The children in the control group who had angular stomatitis at the beginning of the experiment continued to have it all through the experiment.



TABLE IV  
*Clinical results*

Standard	Control group		Experimental group		Standard error	Significance
	Boys	Girls	Boys	Girls		
1. Mean initial weight (kg) ...	17.06	19.80	17.25	20.20	± 2.90	...
Mean final weight (kg) ...	17.462	20.452	18.589	21.354	...	...
Mean change in weight (kg) ...	0.402	0.652	1.339	1.154	± 0.04	P < 0.05
2. Mean initial height (cm) ...	110.943	117.065	110.810	119.838	...	...
Mean final height (cm) ...	111.67	117.974	112.491	121.655	...	...
Mean change in height (cm) ...	0.727	0.909	1.681	1.817	± 0.2407	P < 0.05
3. Mean nutritional deficiency score						
initial score ...	9.000	9.142	9.642	9.000	...	...
Final score ...	9.714	10.50	5.642	5.541	...	...
Change in score ...	+0.714	+1.458	-4.000	-3.459	± 3.01	P < 0.05
4. Mean initial hip width (cm) ...	17.600	18.421	17.328	18.170	...	...
Mean final hip width (cm) ...	17.814	18.735	17.92	19.041	...	...
Mean change in hip width (cm) ...	0.214	0.314	0.592	0.871	± 0.03	P < 0.05
5. Mean haemoglobin (g/100 ml)						
Blood—Initial ...	10.61	10.54	9.05	8.18	...	...
,, —Final ...	9.36	9.60	11.41	11.43	...	...
,, —Change ...	-1.25	-0.94	2.36	3.25	± 1.13	P < 0.05
6. Mean red blood cell count (10 <sup>6</sup> /cu mm)						
Initial count ...	3.34	2.95	2.88	2.50	...	...
,, Final count ...	2.28	1.96	3.82	3.88	...	...
,, Change in count ...	-1.06	-0.99	-0.94	1.38	± 0.8814	P < 0.05
7. Mean blood proteins						
Initial readings { Total protein ...	7.410	7.068	7.074	5.366	...	...
Albumen ...	2.687	2.810	2.646	2.466	...	...
Globulin ...	4.723	4.258	4.428	2.900	...	...
Ratio ...	1 : 1.75	1 : 1.51	1 : 1.67	1 : 1.17	...	...
Final readings { Total protein ...	7.564	8.272	10.226	8.151	...	...
Albumen ...	2.789	3.154	4.695	3.908	...	...
Globulin ...	4.775	5.118	5.573	4.243	...	...
Ratio ...	1 : 1.71	1 : 1.62	1 : 1.17	1 : 1.08	...	...

The supplements were realised at the beginning of the experiment but by the seventh week the children receiving the protein supplement did not relish it. None of the children complained of digestive upsets during the experimental period.

*Growth:* There was statistically significant difference in the weight gain made by the experimental group as compared with the control group ( $P < 0.05$ ). The control girls made greater gain than the control boys while the reverse was true in the experimental group. The average weight gain of the controls and experimentals is almost the same as that obtained by Subrahmanyam *et al.*<sup>5,7,13</sup>.

The difference in height between the control and the experimental groups was significant ( $P < 0.05$ ). The average gain in height made by both control and experimental groups is less than that observed by Subrahmanyam *et al.*<sup>5,7,13</sup>. This may be due to a difference in the length of experimental period.

It was also found by statistical analysis that the difference observed between the experimental and control groups could be attributed only to the supplements and not



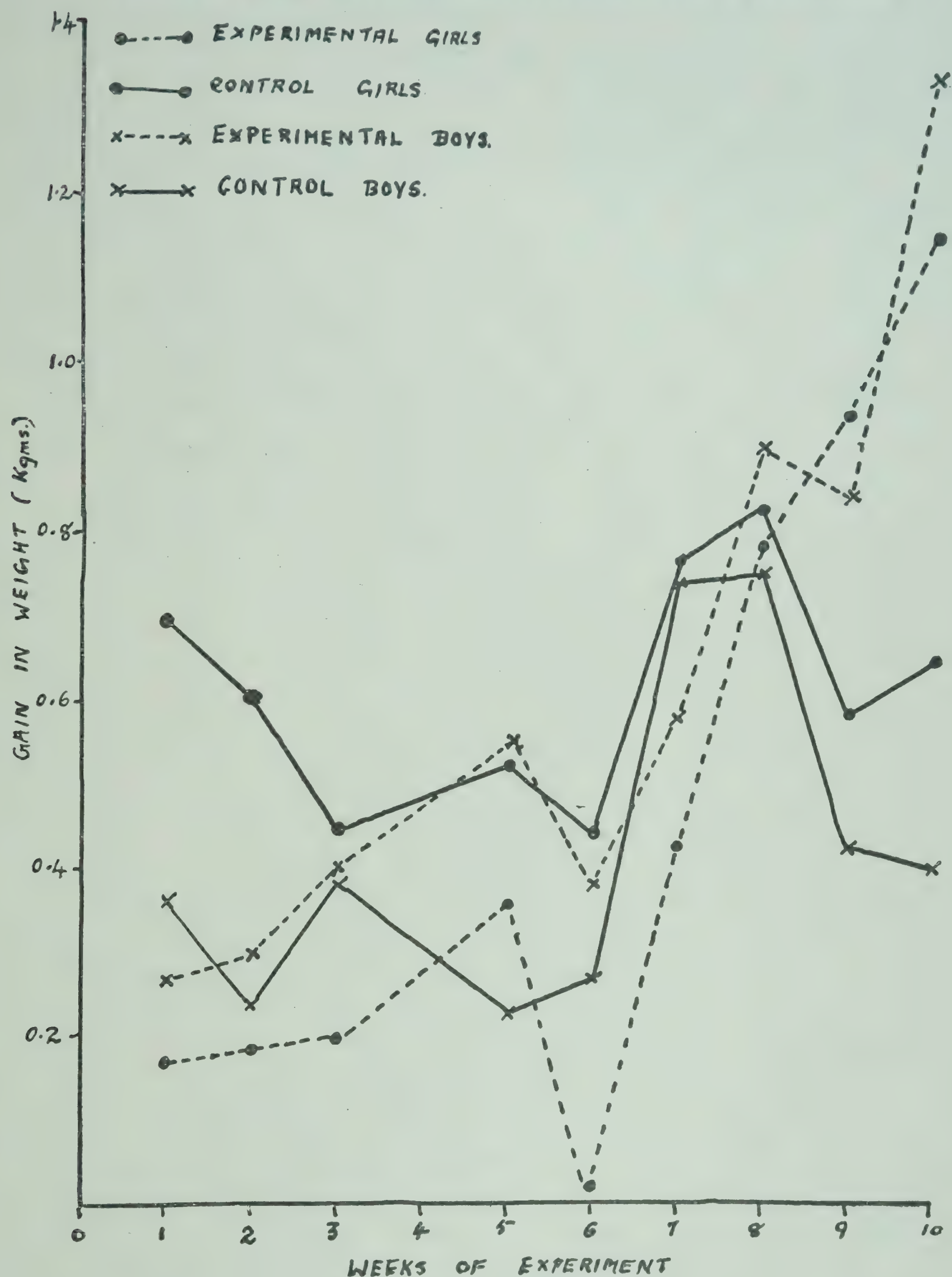


FIG. 1. Effect of a composite protein food on the gain in weight of school children

to the sex difference. This finding is in accord with the work of Cawley and co-workers<sup>17</sup> who report that pubertal acceleration in growth appears from the 9th to 12th year in girls and from the 12th to the 15th year in boys.

Figure 1 shows a depression in the weight of the children between the 4th and the 6th week in both the experimental and control groups. After the 6th week the weight



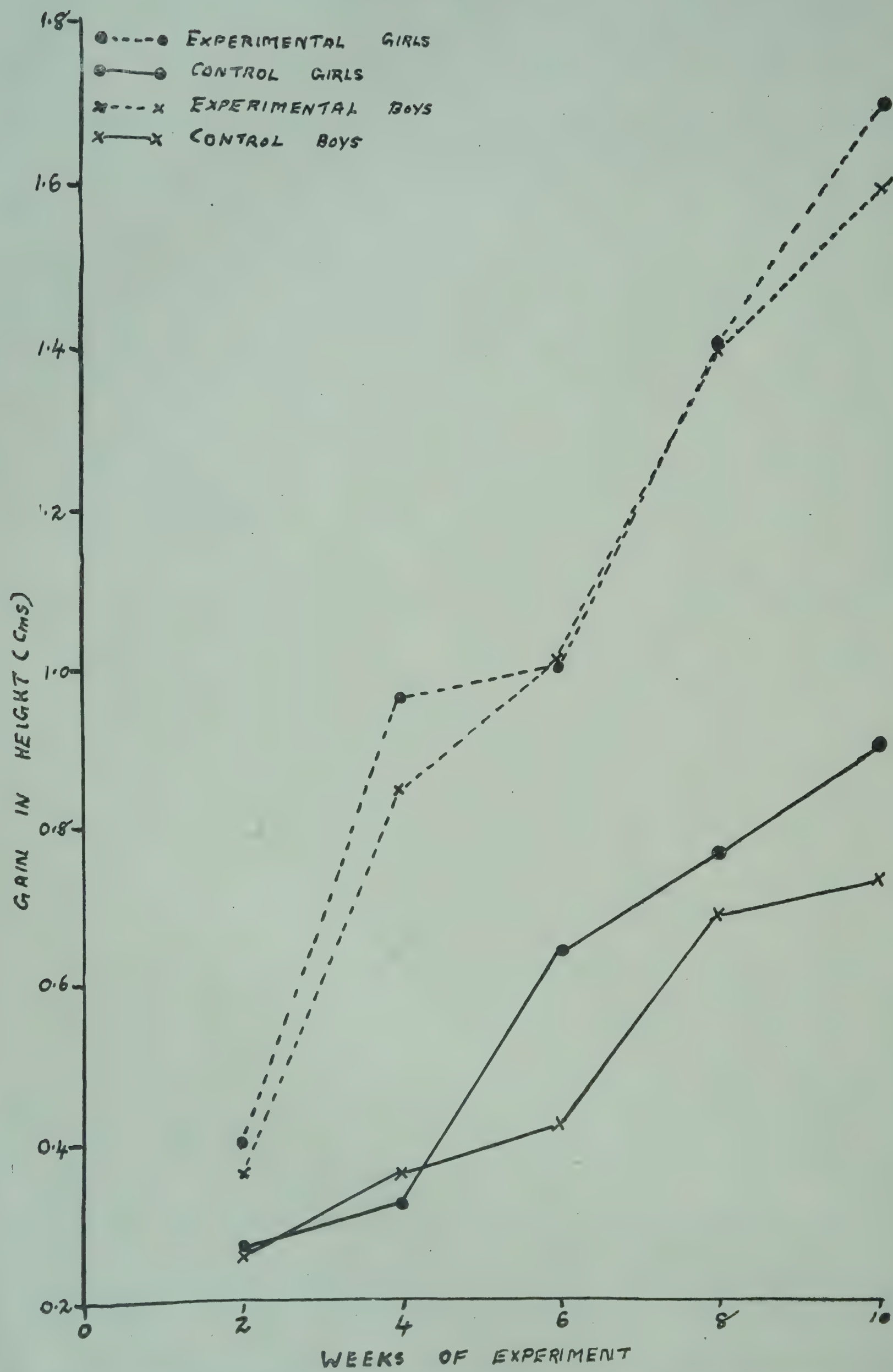


FIG. 2. Effect of a composite protein food on the gain in height of school children



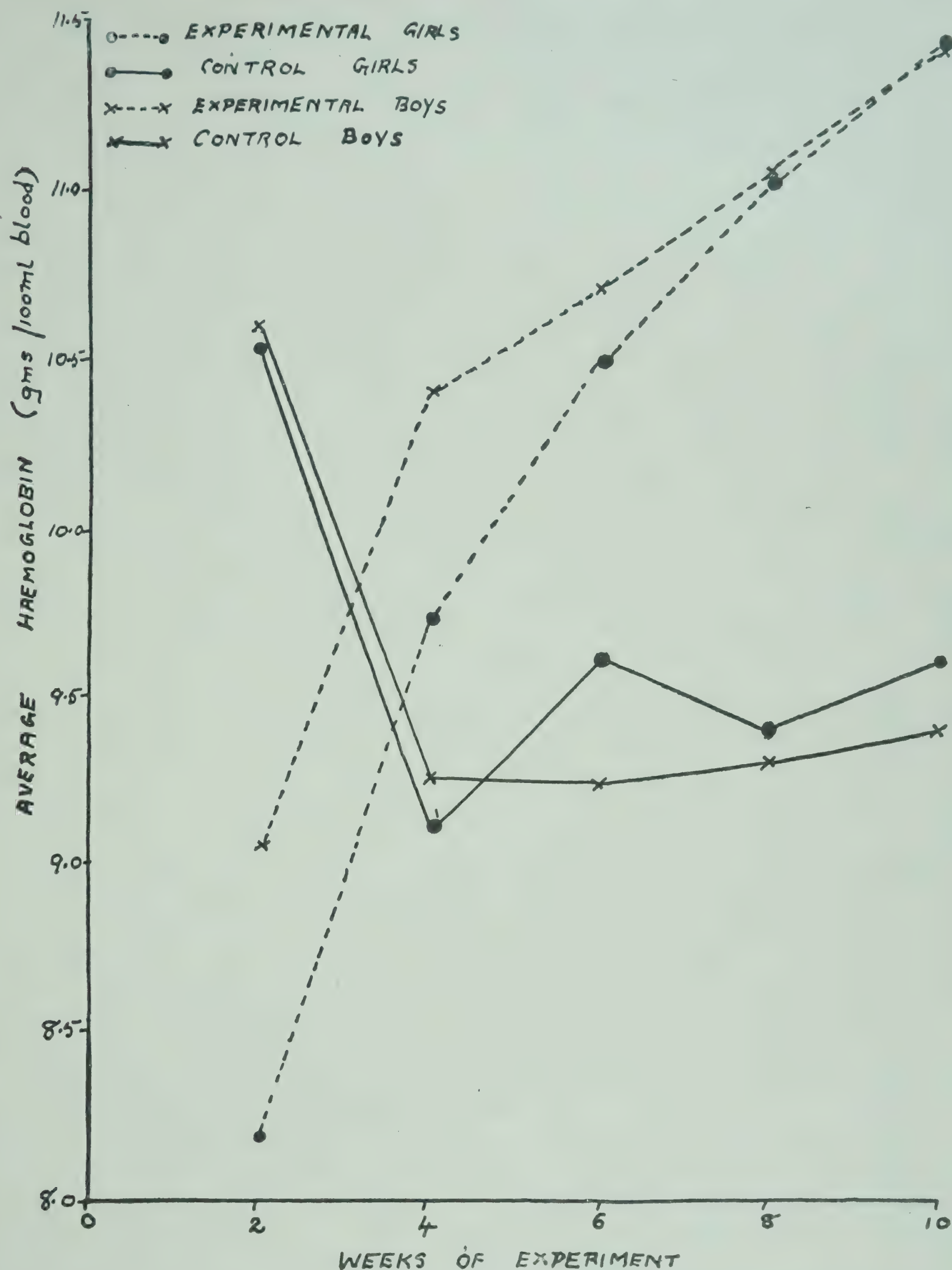


FIG. 3. Effect of a composite protein food on the percentage haemoglobin of school children

curves of the experimental group go up steadily while in the control group there is a rise in weight from the 6th to 8th week and a fall in weight from 8th to the 10th week. The depression in weight between the 4th and 6th weeks may be related to the change in the basal diet. During that period American corn in the basal diet was replaced by



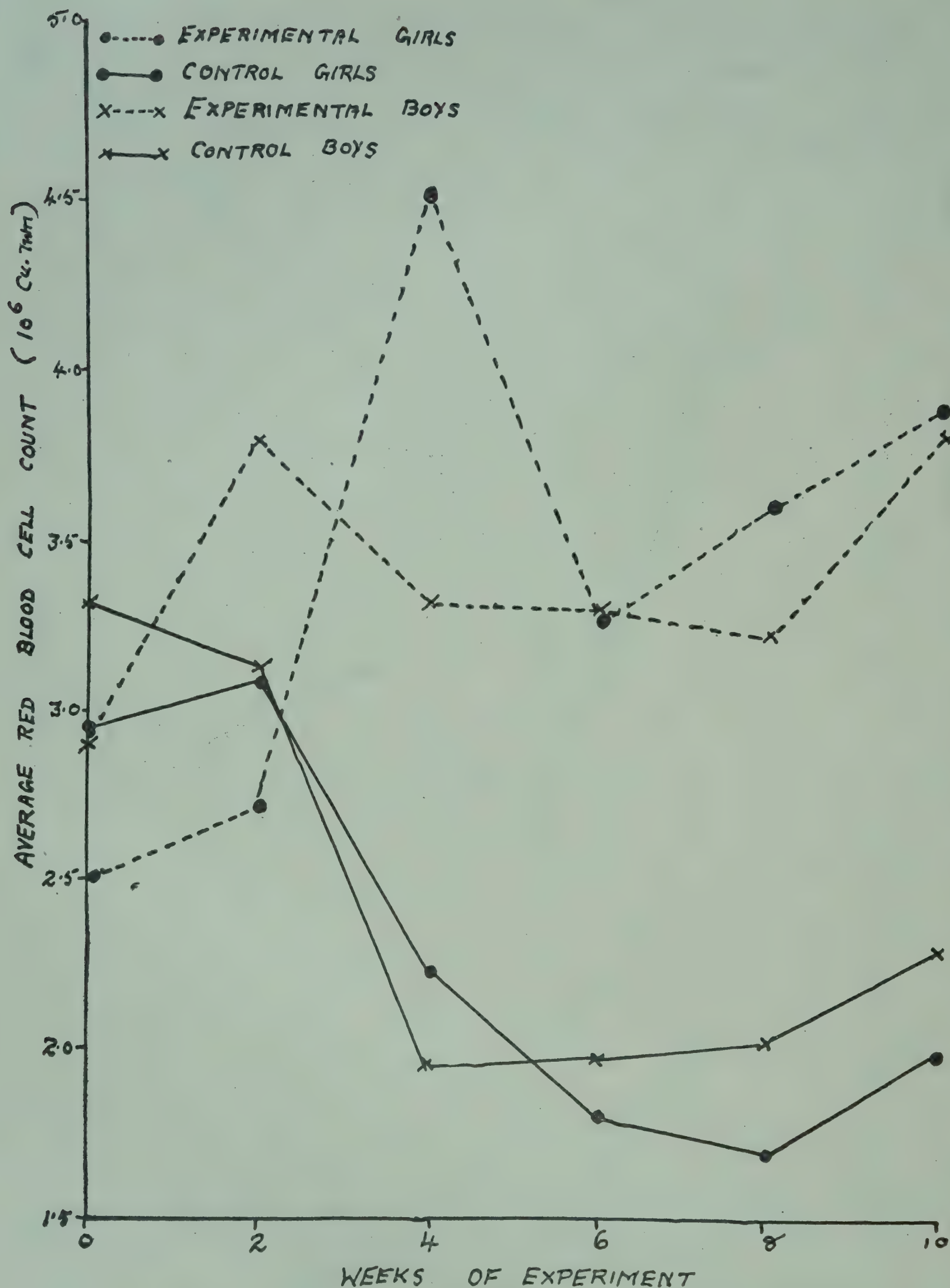


FIG. 4. Effect of a composite protein food on the red blood cell count of school children

rice, wheat or ragi. The rise in weight after the 6th week may be due to the regular use of ragi in place of rice or wheat. It was observed that towards the later stages of experiment none of the children consumed the total quantity of ragi gruel which was mixed with milk or curds prepared from skimmed milk powder. This might explain the depression



in weight observed in the control groups during the last two weeks. In the experimental group the protein supplement might have helped to overcome this depressing effect on growth resulting from the incomplete consumption of the ragi gruel.

*Hip width:* The hip width of boys and girls in this experiment was much below the normal for American children, a finding similar to that of Aykroyd and Rajagopal<sup>18</sup>. The girls in both the experimental and control groups showed a greater increase in hip width as compared to boys although the difference was not significant ( $P < 0.05$ ). Statistical analysis showed that the difference in hip width between the control and experimental groups was significant ( $P < 0.05$ ). It is not possible to discuss these data as hip width measurements were not used by other workers as a standard for judging nutritional status.

*Nutritional deficiency score:* The nutritional deficiency score of the experimental girls and boys decreased by  $-4.00$  and  $-3.45$  respectively while in the control group there was an increase of  $1.458$  for girls and  $0.714$  for boys. These results are almost similar to that of Subrahmanyam *et al.*<sup>5</sup> who observed a nutritional deficiency score of  $-0.15$  for the experimental group and  $1.48$  for the controls in a study of the effect of vegetable milk curd on children.

The teeth of most of the children (75 per cent in both groups) were found to be coated with a thick yellow or greenish black deposit. Though it was the opinion of the dentist that this was due to bad hygiene and breathing through the mouth, the work of Potjieter<sup>19</sup> shows a definite correlation between dental health and the diet of children. There is some suggestion that changes in the pH of the oral cavity brought about by a faulty diet can also give rise to such deposits.

*Blood picture:* A significant increase ( $P < 0.05$ ) was found in the red blood cell count and percentage haemoglobin of the experimental group. A decrease in the red blood cell count and percentage haemoglobin was observed in the controls. The increase in the red blood cell count and percentage haemoglobin in the experimental group in this study was higher than that observed by Subrahmanyam *et al.*<sup>5,7,13</sup>. This may be due either to the initial level of haemoglobin and red blood cell count being lower in these children as compared with observations reported by other workers or to the effect of the supplement itself. The initial mean values for the red blood cell count and percentage haemoglobin observed by Subrahmanyam *et al.*<sup>5</sup> ranged from  $4.13$  to  $4.53$  and from  $11.20$  to  $11.57$  respectively. In this experiment the initial red blood cell count range was from  $2.50$  to  $3.34$  and the percentage haemoglobin was from  $8.18$  to  $10.61$ .

Figure 4 shows a rise in the red blood cell count of the experimental boys at the second week and of the experimental girls at the fourth week. This is due to the higher red blood cell count of 3 boys ( $4.86$ ,  $6.4$  and  $4.94$ ) at the second week and of 2 girls ( $5.24$  and  $6.9$ ) at the fourth week. One of the 3 boys and both the girls were having a fever and sore eyes at the time.

*Blood proteins:* None of the children had a normal albumen/globulin ratio<sup>14</sup> of  $2:1$  at the beginning of the experiment. The initial mean ratio varied from  $1:1.17$  to  $1:1.75$ . Analysis of blood proteins at the end of the experiment showed very slight changes in the albumen/globulin ratio of all the children. The albumen/globulin ratio of the control boys changed from  $1:1.71$  to  $1:1.72$  and that of the control girls from  $1:1.15$  to  $1:1.62$ . The ratio of the experimental boys changed from  $1:1.67$  to  $1:1.17$  and that of the experimental girls from  $1:1.17$  to  $1:1.08$ . Although the experimental children did not show a normal albumen/globulin ratio the change was in favour of the



protein supplement. However, marked differences in blood proteins cannot be expected as the experimental period was comparatively short.

The data obtained from this study are very limited. Further quantitative data could be obtained by studying the effect of this supplement on a larger number of school children belonging to different age groups for a longer period.

### Summary

A feeding experiment extending over a period of ten weeks was carried out on 42 children (both boys and girls) between 7 and 9 years of age in an orphanage in Madras city. The purpose of the study was to assess the value of supplementing the school lunch with 1 oz of roasted bengal gram dhal, 1 oz of groundnuts and 1 oz of skim milk powder. Data regarding the weight, height, haemoglobin level and red blood cell count were obtained periodically during the course of the experiment. Data regarding the nutritional status, albumen/globulin ratio and hip width were obtained once at the beginning and once at the end of the experiment. Statistical analysis of the results showed a significant improvement ( $P < 0.05$ ) in the weight, height, hip width, nutritional status, percentage haemoglobin and red blood cell count of the children receiving the supplement over those in the control group receiving an iso-caloric placebo. An improvement in the albumen/globulin ratio was observed in the experimental group over those in the control group even though the levels in both groups did not reach the normal values.

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# INFLUENCE OF VARIETY AND CULTURAL CONDITIONS ON THE PROTEIN CONTENT IN FOODSTUFFS

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The protein content of cereals is largely dependent on variety, climate, soil type, fertilizers, crop rotation and other agricultural practices. Information on the protein content of Indian wheats and the influence of different factors thereon is rather meagre. Work on the survey of wheat protein was initiated at Lyallpur (Punjab). A number of wheat samples grown in different parts of India were analysed for their protein content. Subsequently work of this nature was taken up at I.A.R.I. The protein contents of some important varieties of Indian wheats were found to vary from 8.7 to 17.4 per cent<sup>1,2,3</sup>.

In view of such large variations in the protein content of Indian wheats, a systematic study was taken up at I.A.R.I. under a scheme sponsored by the Indian Council of Agricultural Research (1953-57). In this Scheme fourteen varieties of Indian wheats were grown in three replications with identical manurial treatment both at Delhi and at Pusa (Bihar) over a period of three consecutive years and were analysed for their protein and other constituents. The protein content of these wheats grown at Delhi varied from 8.8 to 11.2 per cent in 1953, 9.4 to 12.1 per cent in 1954 and 7.6 to 10.4 per cent in 1955. The protein content of those grown at Pusa (Bihar) during the corresponding years varied from 9.2 to 12.3 per cent, 10 to 13.8 per cent, and 11.4 to 14.6 per cent respectively.

The data when analysed statistically by pooling the results for the two places over three years, revealed certain interesting facts with respect to the effect of variety, place and season on the content of protein. The average varietal differences were highly significant.

The varietal differences were also highly significant when tested against the interaction between varieties and year. This would mean that apart from seasonal variations, there are definite variations in the protein content of different varieties. On the average, the protein content was significantly higher at Pusa than at New Delhi. The interaction between places and years was also significant. The average protein content in the three years differed significantly from each other. The highest value was obtained in the second year while the lowest was obtained in the first year.

## **Variation among varieties at individual places**

The interaction between varieties and places was highly significant. It was found that NP 761, NP 770 and NP 775 scored the first three places at Delhi, while NP 4, NP 761 and NP 770 scored the same at Bihar.

*Gluten content:* In view of the special importance of gluten among wheat proteins, a number of pure strains of Indian wheat were analysed for their gluten content. It was found to vary from 6.8 to 11.3 per cent<sup>2,3,4</sup>.



Wheats grown in tri-replicates under identical manurial treatment at Delhi and at Pusa (Bihar) over three consecutive years showed a large variation in gluten content. The average varietal difference in the content of wheat gluten was highly significant. The average gluten content was also found significantly higher at Pusa than at Delhi.

*Amino acids in wheat:* There appears to be considerable variation in the content of amino acids in different wheats<sup>5</sup>. Some Indian wheats were found to contain 0.22 to 0.34 per cent of methionine<sup>6</sup> and 0.3 to 0.37 per cent lysine<sup>7,8,9</sup>.

*Protein efficiency ratio:* Protein efficiency ratio of a few pure Indian wheats grown at I.A.R.I. was found to vary from 1.75 to 1.89 at 8 per cent protein level.

*Effect of fertilizers:* The beneficial effect of nitrogenous fertilizer on the protein content of wheat has been observed by several workers. Wheat protein has been found to increase in grains raised with ammonium sulphate, green manure and with a legume in rotation<sup>10,11,12,13</sup>. The increase of grain protein as a result of late application of nitrogen by spraying urea on wheat leaves has also been observed<sup>14, 15, 16</sup>. Phosphatic fertilizers, on the other hand, tend to depress the protein content of the grain<sup>13,17</sup>.

Besides the major nutrients, micro-nutrients have also been reported to influence the content of grain protein. Treatment of the soil with either zinc or manganese resulted in a significant increase in the protein content of wheat, the maximum increase being with the manganese treatment while foliar application of zinc, copper and magnesium, but not manganese, showed similar effect<sup>18</sup>.

*Pulse proteins:* Much work has already been done on pulse proteins in India<sup>19, 20, 21, 22</sup>. Some work on pure strains of pulses was taken up at this Institute. Strain variation in protein content was found in the case of peas, lentil, and mung. Significant strain variation with respect to PER was also observed in pea, lentil and green gram<sup>23</sup>.

*Amino acids in pulses:* The contents of methionine, lysine, threonine and tryptophane in arhar and urid dhals were estimated. Urid contains a higher concentration of all the amino acids as compared to those of arhar. The availability of methionine, lysine, threonine and tryptophane present in arhar was also determined. The availability of methionine and threonine was found to be lower than that of tryptophane and lysine<sup>24</sup>. Methionine, cystine and tryptophane content of pure strains of different pulses such as urud, gram, lentil, mung, arhar, pea, khesari, *Vicia sativa*, *Vicia hirsuta* were also analysed. Methionine varied from 0.184 per cent (khesari) to 0.964 per cent (urid). Cystine from 0.052 per cent (mung) to 0.245 per cent (gram) and tryptophane from 0.03 per cent (gram) to 0.125 per cent (*Vicia sativa*)<sup>25</sup>.

### Supplementation with amino acids

The effect of addition of amino acid on the PER and BV of pulse proteins was studied. Methionine supplementation significantly increased the PER as well as the BV of arhar, urid, mung and lentil proteins. Tryptophane and threonine significantly improved the BV of arhar but not that of urud. The same failed to cause any significant increase of the PER value of these two pulses. Lysine significantly increased the BV as well as PER of arhar proteins.<sup>24, 26</sup> Singh *et al.*,<sup>24</sup> Esh and Som<sup>22</sup> observed an increase in PER of Bengal gram and lentil proteins by methionine but not by tryptophane, threonine and lysine.



*Effect of vitamin B<sub>12</sub>:* Supplementation of 10 $\mu$ g of vitamin B<sub>12</sub> had no significant influence on the PER of arhar protein at 12 per cent level while 20 or 30 $\mu$ g of vitamin B<sub>12</sub> showed marked increase. At this level of protein, 20  $\mu$ g of vitamin B<sub>12</sub> also significantly increased the BV of arhar proteins. At 17 per cent protein level, vitamin B<sub>12</sub> showed no effect either on PER or BV of arhar<sup>24</sup>. The BV of lentil did not increase by a supplement of 6  $\mu$ g vitamin B<sub>12</sub><sup>26</sup>. Esh<sup>27</sup> reported increase in PER of lentil proteins by vitamin B<sub>12</sub> supplementation at 18 per cent level but not at 12 per cent.

*In vitro digestion of pulse proteins:* Pancreatic digestion of proteins in gram, mung, lentil, arhar, urud and pea was compared with that of meat-meal and casein. The *in vitro* digestion of pulse proteins was found to be much slower than that of meat meal and casein. Among the pulses gram, lentil and pea were more easily digested as compared to those of proteins of arhar, urud and mung.

*Grasses and fodder legumes:* Protein content of twelve legumes and twelve grasses varied considerably ranging from 12.8 to 26.5 per cent and from 5.8 to 20 per cent respectively. Cystine and tryptophane content of these grasses and legumes were low as compared to methionine. Different legumes and grasses differed considerably among themselves in the content of these amino acids.<sup>25</sup>

Investigations were undertaken to study the effect of monthly cuts on the protein and other constituents of ten Indian grasses. Monthly cuts were found to maintain the protein content of different grasses fairly high<sup>28</sup>.

Stage of growth was found to exert a pronounced effect on the protein content and other constituents of grasses. Protein content of the grasses decreased with the advancement in the maturity of the plants<sup>29</sup>.

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# NITROGENOUS SUBSTANCES IN PASSION FRUIT (*PASSIFLORA EDULIS* SIMS.) WASTE

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In studies on the chemical composition of passion fruit<sup>1-4</sup>, its rind (skin) and seeds have been shown to contain 12-15 per cent protein, but there is no published information available on the nature of the protein or the free amino acid make-up in passion fruit rind or seeds. The present report covers these aspects.

## Experimental

*Raw material: (a) Rind:* From a bulk lot of healthy purple passion fruits, 10 lb of the rind were taken, chopped coarsely and then minced through a stainless steel mincer. In order to have a uniform sample throughout these studies, the minced material was freeze-dried in a Stokes 'Freeze Drier' at - 40°F under a reduced pressure of 50  $\mu$ . It took about 3 hours to freeze-dry the rind to about 5.8 per cent moisture content. The freeze dried material was quickly powdered to about 30 mesh in a pulverizer. The material stored at 0°C was used in these studies.

*(b) Seeds:* Seeds were ground, defatted and passed through 60 mesh seive. This material was used throughout these studies.

*Methods of analysis:* Moisture, fat, crude fibre, protein, acidity, tannin, starch, ash, calcium and iron were determined by the A.O.A.C. methods of analysis<sup>5</sup>.

*Fractionation of nitrogen:* Known weights of the freeze-dried material from rinds of both green and fully ripe fruit were successively extracted twice with water, 3 per cent NaCl solution, 70 per cent alcohol and 0.2 per cent NaOH solution for 2 hours each and after centrifugation, the supernatant liquid in each case was removed and made up to known volume. The nitrogen in the various fractions was then determined, as usual. Similar studies were made on the seed powder. The results obtained are presented in Table II.

*Total and non-protein nitrogen:* Total nitrogen was estimated in the rind at two stages of maturity of the fruit and seeds by the micro-Kjeldahl method, while non-protein nitrogen was estimated in the trichloroacetic acid filtrate. The results are given in Table II.

*Free amino acids:* The passion fruit rinds were chopped and blended in a Waring blender for five minutes with sufficient volume of 95 per cent ethanol to give a final concentration of 70 per cent ethanol. For a comparative study, a similar alcoholic extract was prepared from passion fruit seeds as well. The seeds were crushed in a mortar, extracted with 70 per cent alcohol and the extract was concentrated at low temperature under vacuum and stored at low temperature for subsequent studies.



The total nitrogen in the alcoholic extracts was determined by the micro-Kjeldahl method. Portions of the clear supernatant extracts representing about 15 mg of N gave good separation on the chromatogram. Throughout our investigations, circular paper chromatography<sup>6</sup> using n-butanol-acetic acid—water system (4:1:5 V/V) was adopted. Since a single run did not give clear separation, the multiple-run technique was adopted.

Results and Discussion

Chemical composition of rind and air-dried seeds (Table I) revealed that, on dry weight basis, they contained 11.90 and 11.13 per cent of crude protein respectively.

TABLE I  
Chemical composition of passion fruit rind  
(*Passiflora edulis* Sims.)

Physico-chemical characteristics	Rind		Seeds <sup>1</sup> (Air-dried) %
	Fresh weight basis %	Moisture free basis %	
Moisture ... ..	80.0	...	4.4
Ether extract ... ..	0.2	0.8	23.8
Crude Fibre ... ..	6.6	32.9	55.7
Sugars (i) Total sugars (as invert) ...	1.6	8.2	Traces
(ii) Reducing sugars (as invert) ...	1.3	6.7	Nil
(iii) Non-reducing sugars (as sucrose)	0.3	1.4	Traces
Starch ... ..	1.9	9.6	2.6
Acidity ... ..	0.15	0.7	...
Pectin (as Ca pectate) ... ..	2.5	12.4	...
Total astringents ... ..	3.0	14.9	...
(a) Tannins ... ..	1.9	9.6	...
(b) Non-tannins ... ..	1.11	5.3	...
Protein (N × 6.25) ... ..	2.4	11.9	11.1
Total ash ... ..	1.4	7.2	1.8
Calcium ... ..	0.1	0.3	0.1
Iron (mg/100g) ... ..	6.1	30.6	18.00
Phosphorus ... ..	0.02	0.1	0.64
Ascorbic acid (mg/100g) ... ..	82.9	414.45	...

<sup>1</sup> Calculated

Studying the effect of salt concentration on the extractability of nitrogen from rind, it was shown that about 64.4 per cent of the total nitrogen was extractable (with 2-3 per cent salt solutions). Studies on the fractionation of nitrogen revealed that, of the total nitrogen in ripe rind, albumin, globulin, prolamine and glutelins comprised 49.83, 15.71, 2.96, and 7.90 per cent respectively (Table II). About 23.6 per cent of the total nitrogen was unextractable.

Comparing the two stages of maturity, (i) prolamines and glutelins were absent at the green stage, (ii) albumin was slightly higher at the green stage than at the ripe stage and (iii) globulin was less than that at the ripe stage.



TABLE II  
*Fractionation of nitrogen in passion fruit rind*

Nitrogen fractions*	% N in Rind				Seeds	
	Stages of maturity		As % of total nitrogen		% N	% of total N
	Green	Purple	Green	Purple		
Total nitrogen %	2.80	1.91	...	...	8.51	...
Non-protein nitrogen %	1.32	1.25	47.10	56.40	0.65	7.67
Albumin (Water soluble %)	1.58	0.95	56.40	49.74	0.71	8.35
Globulin (3 % NaCl)	0.10	0.30	3.68	15.71	0.76	8.93
Prolamine (alcohol sol. %)	Nil	0.06	...	2.88	0.06	0.70
Glutelins (alkali sol. %)	Nil	0.15	...	7.90	3.20	37.60

\* All figures calculated on dry-weight basis

*Free amino acids in rind:* As judged by position characteristic colour of the spots with ninhydrin, chromatography with known amino acids and spot tests<sup>7-11</sup>, the presence of the following amino acids in passion fruit rind and skin was indicated.

TABLE III  
*Distribution of free amino acids in passion fruit skin and seeds*

Amino acids				Skin	Seeds
Leucines	...	...	...	++	+
Valine	...	...	...	+	Trace
Tyrosine	...	...	...	+	+
Proline	...	...	...	++	Trace
Threonine	...	...	...	+	Trace
Glycine	...	...	...	+	+
Aspartic acid	...	...	...	+	+
Arginine	...	...	...	+	+
Lysine	...	...	...	Trace	Trace

*Free amino acids in seeds:* Studies on the free amino acids in seeds also revealed that they contained the same amino acids as found in rind except that in seeds proline occurred in traces. Likewise, there was only a difference in the relative concentration of different amino acids.

Comparing passion fruit skin and seeds with other commercially important fruits<sup>12</sup>, it may be interesting to note that in amla, banana, green guava, Coorg mandarin orange and pineapple, proline was not present in detectable amount and except in banana, leucines too were not present in detectable amounts in the above fruits. However, as in other



fruits, methionine, phenylalanine and tryptophane were also not detected in passion fruit skin and seeds.

## Summary

Passion fruit rind (skin) and seeds comprising respectively 50 and 15 per cent of the whole fruit, are a fairly good source of protein (12-15 per cent). In ripe *Passiflora* fruit rind, albumin, globulin, prolamine and glutelins comprised 49.83, 15.71, 2.96 and 7.9 per cent respectively of the total nitrogen (1.91 per cent). In seeds, the corresponding figures were 8.35, 9.93, 0.70 and 37.60 per cent respectively. About 23.6 per cent of the total nitrogen was not extractable in rind and 36.75 per cent in seeds.

Comparing the two stages of maturity of the fruit, it was found that prolamine and glutelins were absent at the green stage while albumin content was slightly higher at the green stage than at the ripe stage.

Paper chromatographic studies in conjunction with suitable chemical tests revealed that both passion fruit rind and seeds contained nine free amino acids, namely leucine, isoleucine, valine, tyrosine, proline, threonine, glycine, aspartic acid, arginine, and lysine. However, in seeds, proline occurred in traces.

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# AMINO ACID COMPOSITION OF LARVAE OF THE POTATO MOTH (*GNORIMOSCHEMA OPERCULELLA* ZELL.) AND THEIR NUTRITIVE VALUE FOR REARING PARASITES

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*Gnorimoschema operculella* Zell. though a pest of potato tubers under field and storage conditions, is also a beneficial insect in the sense that it is being widely used in its different stages as an alternate host for a number of useful hymenopterous parasites like *Bracon*, *Microbracon*, *Trichogramma*, *Apanteles* and many others. These parasites are valuable tools in the field of biological control of lepidopterous cotton borers against which all modern methods of chemical control have so far been of no value. For a full exploitation of their parasitising potentialities it is necessary to understand the host-parasite relationship with reference to their nutritional requirements. More information on this aspect will be of direct value in devising synthetic non-biotic media for rearing parasites outside the body of the host and in the study of nutritional physiology.

## Material and Methods

A culture of *Gnorimoschema operculella* was maintained in the laboratory in 10 lb glass jars containing sorghum and dried brewer's yeast at 29°C and 60-65 per cent R.H. About 30 healthy third instar larvae were taken, freed from sticking flour particles and starved for 48 hours. They were then dissected in distilled water so as to remove guts and their contents. The remaining tissues were digested in 10 ml of 6 N HCl at 110°C for 24 hours to hydrolyse the proteins. After 24 hours the material was filtered and the filtrate evaporated under vacuum. It was then diluted with distilled water and dried under reduced pressure. This process was repeated several times in order to remove excess acid.

The hydrolysate was analysed for its amino acid contents by paper partition chromatography<sup>2</sup>.

## Results and Discussion

The hydrolysed tissues of larvae consist of at least 17 amino acids, viz., aspartic acid, glutamic acid, cystine, serine, glycine, threonine, alanine, tyrosine, valine, methionine, proline, histidine, lysine, arginine and leucine-isoleucine. There appear to be three more spots which could not be identified. Their identity can only be established by carrying out further investigations. Tryptophan which is an important and universally occurring amino acid in the plant and animal kingdom is not represented because of its extreme susceptibility to acid hydrolysis which destroys it. Pant, Gupta and Nayar<sup>5</sup> reported similar types of amino acids in *Corcyra*. Moorefield and Fraenkel<sup>4</sup> found 14 amino-acids, 2 unidentified substances and cystic acid and glucosamine in the salivary secretion of



*Phormia*. Micks and Ellis<sup>3</sup> reported similar types of amino acids occurring in free state in different stages of mosquito. They also found that concentration of amino acids varied with stages, least being in pupae. The amino acid component of insect body protein seemed to have qualitative pattern similar to that found in some vertebrates<sup>1</sup>.

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# AUTHOR INDEX

<b>A</b>					
Abraham, K. A.	37	Balasundaram, S.	374	Bhandarkar, A. P.	380
Acs, G.	325	Baliga, B. R.	335	Bhagvat, K.	362
Adams, D. H.	123	Ball, C. D.	362	Bhatt, R.	73
Addis, T.	139	Baltus, E.	89	Bhargava, P. M.	37
Adler, E.	210	Bancroft, R. W.	146	Bhat, J. V.	212, 216
Agarwala, S. C.	325	Bandurski, R. S.	193	Bhatia, D. S.	54, 260, 264, 269, 270, 274, 275, 284, 300, 301, 368, 430, 369
Ahmed	132	Banerjee, B. W.	258	Bhatnagar, S. P.	433
Akabori, S.	311	Banerjee, S. K.	317	Bhuvaneshwaran, C.	90
Albaum, H. G.	166	Banerjee, N. D.	258	Bhaugat, S. B.	123
Alexander, K. M.	440	Banerjee, S.	124, 128, 344, 355, 356	Bhattacharya, R. K.	107
Alderton G.	58	Bang	69	Bhattacharya, R. N.	59
Allegri, A.	123	Banhieni, L. L.	122	Bianconi, A. M.	167
Allen, B. K.	82	Bangham, A. D.	19	Bidwell, R. G. S.	237
Allison, C. A.	19	Bannerjee, B. W.	258	Biley, J.	300
Allison, J. B.	146, 166	Bannerjee, R. M.	433, 434	Bird, E. J.	311
Almquist, H. J.	377, 380	Bannerjee, S.	203, 355, 356	Bird, H. R.	146, 153
Alpen, E. L.	247	Banerjee, S. K.	317	Bissett, H. M.	295, 300
Altschul, A. M.	264	Bardawill, C. J.	123, 166, 210	Biswas, D. K.	128
Ambe, S. K.	300, 380	Barnafi, L.	180	Biswas, T. D.	433
Amies, C. R.	196	Barner, H. O.	87, 89	Bjork Walter	29
Amiya, B.	130, 179	Barnes, R. H.	76, 146, 350, 354	Black, A.	146
Ammerman, C. B.	122	Barton, E.	167	Blackburn, S.	66
Amos, H.	108	Barton-Wright, E. C.	166, 355, 356	Blix, G.	193
Anand, N.	106	Basak, R.	128	Block, J. R.	89
Anantharaman, K.	54, 260, 264, 363, 393	Basinski, D. H.	128	Block, R. J.	99, 216, 247, 300, 345, 352, 353, 368, 374, 438
Anastel, J. H.	106	Basu, D. K.	187	Boas, N. F.	66
Anderson, P. R.	49	Basu, K. P.	345, 353, 433	Bocobo, D. L.	216
Anson, M. L.	46	Basu, N. M.	112	Bodansky, O.	112
Appaji Rao, N.	226	Basu, U. P.	362, 404	Boisselot, J.	139
Appel, P.	6	Bateman, I.	112	Bolling, D.	216, 300, 438
Appel, W.	378, 380	Baumann, E.	66	Boman, H. G.	29
Appleton, H. D.	202	Baumann, C. A.	112, 166	Bonnet Maury, P.	99
Apte, U. V.	380	Beach, E. F.	368	Bonner, J.	253
Apter, R. A.	99	Beck, L. V.	167	Bonner, W. D.	112
Archibald, W. J.	6	Becker, D. E.	122	Bolin, D. W.	334
Armbruster, G.	334	Beeson, W. M.	334	Bollet, A. J.	193
Arnrich, L.	112	Begwood, E. J.	128	Bose, A. N.	326, 397, 404
Arnstein, H. R. V.	77, 82, 167	Behal, F. J.	185	Bose, A.	421, 430
Arullani, C.	185	Behar, M.	374	Bosshardt, D. K.	76, 146
Asarkh, R. M.	210	Bell, P. H.	180	Bothwell, J. W.	112
Ashbel	171	Bencze, W. L.	24	Boucek, R. J.	193
Autret, M.	289, 295, 301	Bendall, J. R.	328	Bowen, J. J.	247
Axelrod, A. E.	122, 123	Bender, A. E.	139, 301, 353	Bowland, J. P.	146, 153
Aykroyd, W. R.	421, 429, 430	Ben Ishai, R.	99	Boxer, G. E.	166
Ayres, M. M.	76	Benfey, B. J.	180	Boyden, S. V.	225
		Bennet, J. P.	253	Boyer, P. D.	238
		Berg, R. T.	146, 153	Brachet, J.	83, 89
		Bermes, E. W.	403, 410	Bradley, S. E.	247
		Bernstein	69	Brand, E.	66
		Bernheim, F.	106	Bratzler, J. W.	146
		Berger, S.	112	Braunstein, A. E.	123, 210
		Bergeim, O.	139	Bressani, R.	374
		Berenbeim, M. W.	171	Bird, E. J.	311
		Bessey, O. A.	166	Briggs, G. M. (Jr.)	99
		Bethke, R. M.	433	Brink, N. G.	180
		Beveridge, J. M. R.	295	Brodie, B. B.	202
		Beuk, J. F.	334	Brody, S.	174
		Bhagavan, H. N.	129	Brown, F. R. (Jr.)	139
		Bhagwan, R. K.	275, 289, 374, 393, 396, 430		



- Brown, A. H. 82  
 Brown, A. K. 193  
 Bruff, J. B. 106  
 Brunngraber, E. G. 193  
 Bullock, K. 49  
 Bundy, H. E. 24  
 Burch, H. B. 123  
 Burmes, E. W. 414  
 Burrows, W. 196  
 Burris, R. W. 174  
 Burris, R. H. 89, 139, 166  
 Burtner, H. J. 414  
 Burton, K. 82  
 Buschke, W. 122  
 Butler 69
- C**
- Cabannes, R. 19  
 Calloway, D. H. 146  
 Cama, H. R. 8, 15, 19, 112, 226, 363, 368, 374  
 Campagnari, F. 123  
 Campbell, J. A. 290, 295  
 Campbell, G. F. 378, 380  
 Caneva, G. 202  
 Carey, J. E. 29  
 Cary, C. A. 76  
 Carpenter, K. G. 123  
 Carpenter, K. J. 300  
 Carter 69  
 Carter, N. M. 328  
 Caspersson, T. 83, 89  
 Castillo, R. 295  
 Catherine, P. D. 317  
 Chakravarthi 132  
 Chakravarti, N. K. 404  
 Chakravorty, P. R. 257, 258  
 Chalam, M. C. 374  
 Champlin, M. 433  
 Chandrasekhar, N. 19  
 Chandrasekhara, M. R. 433  
 Chang, I. C. L. 374  
 Chang, V. T. 76  
 Chantrene, H. 99  
 Chapman, D. G. 295  
 Chargaff, E. 99  
 Charkey, L. W. 76, 167  
 Chatterjee, G. C. 197  
 Chatterjee, K. K. 405  
 Chatterjee, K. P. 128, 356  
 Chatterjee, T. K. 258  
 Chattopadhyay, H. 356  
 Chaudhuri, K. C. 405  
 Cheldelin 317  
 Chen, L. 167  
 Chibnall, A. C. 237  
 Chick, H. 289  
 Chitre, R. G. 362, 433  
 Choudhury, S. 174  
 Choudhury, A. 168  
 Chowdhury, L. M. 362  
 Christian, C. L. 36  
 Christian, W. 58, 210  
 Chung, D. 46  
 Church, M. B. 311  
 Cifonelli, J. A. 193
- Circle, S. J. 368  
 Clandinin, D. R. 295, 433  
 Clark, E. P. 383, 387  
 Clarke, I. 123  
 Clayton, R. K. 97, 99  
 Cochrane, W. A. 185  
 Cohen, E. 66  
 Cohen 132  
 Cohen, S. S. 87, 89  
 Cohn, M. 106  
 Cohn, C. 146, 387  
 Colemann, V. R. 106  
 Collier, H. B. 123  
 Collins, F. D. 112  
 Collip, J. B. 383, 387  
 Colman, D. 153  
 Colowick, S. P. 203  
 Combo, G. F. 153  
 Comb, D. G. 193  
 Combs, A. M. 123  
 Combs, G. F. 146, 153  
 Common, R. H. 300  
 Conner, R. T. 166  
 Consden, R. 225, 359, 362, 440  
 Constantinidea, P. 171  
 Copeland, D. H. 167  
 Cox, H. R. 139, 153  
 Crane, R. F. 193  
 Creaser, E. H. 99, 106  
 Crompton, E. W. 300  
 Cunningham, L. 166  
 Czaczkes, J. W. 122
- D**
- D'Abramo, F. 193  
 Dabsch, V. M. 301  
 Danske, L. M. 146  
 Dansky, L. M. 153  
 Dandliker, W. B. 46  
 Darmady, E. M. 430  
 Das, N. B. 210, 431, 433, 434  
 Das, B. R. 20, 24  
 Davenport, S. G. T. 430  
 David, M. M. 166, 123, 203, 210  
 Davidson, C. S. 404  
 Davies, J. N. P. 301, 396, 414  
 Davis, B. S. 78, 82  
 Davis, B. D. 100, 106  
 Dawson, R. M. C. 202, 203  
 Day, F. J. 82  
 De, N. N. 179  
 De, N. K. 112  
 De, S. S. 353, 362, 378, 380, 433  
 De, T. S. 357, 362  
 Dean, R. F. A. 289, 301, 374, 396, 414, 418, 420  
 Deb, C. 128  
 Defreitas, A. S. W. 300  
 Dekock, P. C. 253  
 Derrien, Y. 66  
 Desaulles, P. A. 193  
 Desikachar, H. S. R. 76, 362  
 Desai, B. G. 362, 433  
 Desai, D. B. 362  
 Desai, G. B. 54  
 Desai, R. F. A. 418, 420
- Deuel, H. J. (Jr.) 146, 295, 341  
 Devor, A. W. 44  
 Dewey, J. (Jr.) 376, 380  
 de Weid, D. 179  
 Dhingra, P. K. 434  
 Dhungat, S. B. 166  
 Diamant, E. J. 123  
 Diansami, Mor, N. A., 167  
 Diansani, M. U. 166, 167  
 Dichek, M. 122  
 Dicks, M. 19  
 Dietrich, L. S. 76, 167  
 Dies, E. J. 275  
 Dinning, J. S. 82  
 Dixon, H. B. F. 180  
 Dische, Z. 166, 193  
 Djang, S. S. T. 362  
 Doolan, P. D. 247  
 Dobos, R. J. 325  
 Doell, B. H. 139  
 Doery, H. M. 29  
 Donaldson, W. E. 146  
 Donaldson, W. F. 153  
 Doniger, R. 99  
 Doraiswamy, T. R. 275, 284, 289, 374, 393, 396, 420, 430  
 Dorfman, A. 193  
 Drew, R. M. 24  
 Dryden, L. F. 76  
 Drury, H. F. 89  
 Dunn, M. S. 247, 363, 365, 368  
 Dunphy, J. E. 193  
 Duckworth, J. 300  
 Dubnoff, J. W. 167  
 Dupuy, H. P. 378, 380  
 Durrum, E. L. 166, 362  
 Dustin, J. P. 128  
 Dutta, S. K. 59, 67, 69  
 Dutta, S. N. 179  
 Dyer, W. J. 328  
 Dye, M. 112
- E**
- Eaton, F. B. 404  
 Eadie, E. J. 166  
 Easterling, L. 300  
 Eapen, K. E. 260, 266  
 Edsall, J. T. 49  
 Edwards, L. C. 193  
 Edwards, R. M. 122  
 Edwards, S. 167  
 Eisgnhev, H. 123  
 Ellinger, G. M. 300  
 Elliot, K. A. C. 378, 380  
 Elliot, R. F. 146  
 Elliot, W. H. 238  
 Ellis, J. P. 440  
 Elson, D. 99  
 Elvehjem, C. A. 76, 99, 112, 122, 123, 139, 146, 153, 167, 352, 380  
 Emerson, G. A. 166  
 Ershoff, B. H. 123  
 Erdos, T. 106  
 Esh, G. C. 107, 357, 362, 433, 434  
 Evans, M. K. 123  
 Ezra Levin 300



- F**
- Fairbarin, D. 203  
 Falk, K. G. 112  
 Farr, A. L. 29, 82, 99, 203, 247, 253  
 Farmer, F. 300  
 Ferrari, V. 123, 166  
 Fatterpaker, P. 140, 167  
 Felch, W. C. 153  
 Felsenfeld, O. 202  
 Fellers, C. R. 295  
 Ferry, E. L. 284, 289, 374, 356, 380  
 Fevold, H. L. 58  
 Fiala, G. 350, 354  
 Field, E. O. 7  
 Ficq, A. 89  
 Firth, J. 76  
 Fischer, D. W. 300, 376, 380  
 Fisher, J. D. 179, 376  
 Fiske, C. H. 193, 203, 231, 238  
 Finn, D. B. 295  
 Finn, R. K. 300  
 Finny, K. F. 433  
 Fitzgerald, R. J. 106  
 Floodgate, G. D. D. 406, 413  
 Folkes J. B. 83, 89  
 Foley, J. B. 66  
 Folkes, J. P. 89, 99, 106  
 Folkers, K. 180  
 Follis, R. H. 122  
 Forbes, E. B. 146  
 Ford, J. E. 82  
 Forker, B. R. 123  
 Forgacs, P. 180  
 Fortune, W. P. 58  
 Foster, E. G. 112  
 Fraenkel, G. 439, 440  
 Frankland, M. 185  
 Fraser, M. 46  
 Fraser, M. J. 77, 167  
 French, H. V. 328  
 French, D. 49  
 French, C. E. 146  
 Fry, W. 89  
 Friedmann, T. E. 210  
 Fris, J. 122  
 Fromageot, C. 58  
 Frost, D. V. 123  
 Fromn, H. J. 238  
 Fukumoto, J. 203
- G**
- Gaarenstroom, 179  
 Gage, B. S. 317  
 Gale, E. F. 82, 83, 89, 90, 99, 106  
 Ganapathy, S. 362  
 Gangstad, E. O. 128  
 Ganguli, N. C. 362, 378, 380  
 Gassner, F. X. 76, 167  
 Gasanov, A. S. 112  
 Geervani, P. 421  
 Geger, E. 146  
 Geiger, E. 295  
 Gerald 171  
 Gersdorff, C. E. F. 13  
 Geschwind, I. I. 179, 180
- Ghani, M. O. 433  
 Ghosal, S. P. 405  
 Ghosh, S. N. 196  
 Giri, K. V. 13, 19, 24, 66, 216, 225, 231, 325, 362, 438  
 Gilfillan, E. W. 166  
 Ginsburg, A. 6  
 Gilbert, G. A. 7  
 Girdhari Lal 435  
 Garbar, P. 36  
 Glass, R. L. 76  
 Glynn, L. E. 325  
 Gofman, J. W. 153  
 Goldblatt, M. W. 46  
 Goldman, D. S. 210  
 Goodwin, J. F. 193  
 Gomori, G. 66  
 Gonashvili, Sh. G. 46  
 Gopalan, C. 289, 374, 387, 392, 396, 420  
 Gopalan, K. K. 260, 363  
 Gordon, A. H. 362, 440  
 Gordon, D. R. 368  
 Gordon, S. A. 253  
 Gor'kova, A. V. 112  
 Gornall, A. G. 123, 166, 203, 210, 225  
 Goryachenkova, E. V. 123  
 Gottfried, S. P. 123  
 Goulden, J. D. S. 82  
 Govindan, T. K. 328  
 Graber, P. 36  
 Gradwohl 210  
 Graham, C. E. 357, 362  
 Gram, C. R. 153  
 Grau, C. H. 363, 365, 368  
 Gray, D. J. S. 317  
 Greipal, M. 185  
 Green, C. 99  
 Greaves, E. O. 334  
 Gribcau, W. F. 368  
 Griffin, A. C. 166  
 Grinnan 69  
 Gross, F. 106  
 Grossi, L. G. 77  
 Grunert, R. R. 166  
 Guggenheim, K. 122, 123, 167  
 Guha, A. 187, 193  
 Guha, B. C. 187, 193, 257, 258, 344  
 Guha, P. K. 397, 404  
 Gulland, J. A. 295  
 Gunnison, J. B. 106  
 Gunter, G. 210  
 Gupta, P. 439, 440  
 Gupta, Y. P. 433, 434  
 Guttman, A. 295, 300, 317  
 Gyorgy, P. 122, 166
- H**
- Hackmann, R. H. 247  
 Haggen, G. E. 210  
 Hagerty, E. B. 146  
 Hahn, F. E. 106  
 Halkier, E. 179  
 Halvorson, H. O. 89, 99  
 Hamburger, J. 247
- Halevy, S. 167  
 Halpern, S. L. 341  
 Hamoir, G. 328  
 Hammersten 69  
 Hamied, K. A. 216  
 Hanahan, D. J. 202  
 Hardin, R. L. 19  
 Harkness, D. M. 166  
 Harrington, C. R. 66  
 Harrington, W. F. 7  
 Harris, I. F. 13  
 Harris, J. I. 180  
 Harris, R. S. 185  
 Harrison, H. C. 167  
 Harris, M. M. 185  
 Harper, A. E. 76, 139, 146, 153, 167, 351, 354  
 Harper, H. A. 247, 351, 354  
 Hart, E. B. 122, 123, 380  
 Hart, J. S. 171  
 Hartley, B. S. 7  
 Hartman, A. M. 76  
 Hartree, E. F. 196, 210  
 Hartshell, S. F. 58  
 Hause, N. L. 352, 354  
 Haurowitz, F. 19  
 Hasserodt, V. 7  
 Hawk, B. P. 404, 430  
 Hawkes, C. D. 146  
 Hawkins, W. W. 123  
 Heagy, F. C. 171  
 Hegsted, D. M. 380  
 Heen, E. 328  
 Heidelberger, M. 66  
 Heinemann, 132  
 Henry, K. M. 167  
 Henderson, L. M. 123  
 Heppel, L. A. 193  
 Herring, V. 153  
 Herisset, A. 112  
 Heroux, O. 171  
 Herr, E. B. 29  
 Hess, G. P. 46  
 Hestrin, S. 112  
 Hewitt, L. F. 49  
 Hier, S. W. 139, 362  
 Hill, R. J. 413  
 Hill, R. L. 46  
 Hill, F. W. 146, 153  
 Hiller, J. 123  
 Hilmoe, R. J. 193  
 Himsforth, H. P. 325  
 Hirs, C. H. W. 19  
 Hockenhull, D. G. D. 406, 414  
 Hogden, C. J. 321  
 Hogeboom, G. H. 58, 166  
 Hogberg, B. 179  
 Hokin, L. E. 325  
 Holdsworth, E. S. 73, 77, 167  
 Holmes, P. 359, 362  
 Hoogland, P. L. 317  
 Hopps, H. E. 106  
 Hopkins 132  
 Horst, W. 243  
 Horvath, A. A. 58  
 Horner, A. A. 112  
 Hospelhorn, V. D. 36  
 Hrubetz, H. C. 295



Hotchkiss, R. D. 89  
 Huggins, C. 36  
 Huisman, T. H. J. 19  
 Hummel, C. 274  
 Humphreys, S. 122  
 Hunt, C. H. 433  
 Hunt, J. A. 19  
 Hutchin, M. E. 247  
 Hutchison, R. 66

## I

Iljin, W. S. 253  
 Inamdar, A. N. 375  
 Indiramma, K. 369  
 Ingram, V. M. 19  
 Ishizaka, K. 36  
 Ishizaka, T. 36  
 Itano, H. A. 19  
 Iyengar, J. R. 274, 300, 301  
 Iyengar, N. V. R. 335  
 Indira, K. 430

## J

Jackson, D. S. 193  
 Jacobs, G. F. 19  
 James, W. H. 146  
 Jawetz, E. 106  
 Jayalakshmi, V. T. 386, 387  
 Jencks, W. P. 166  
 Jennings, R. K. 196  
 Jenson, E. V. 36  
 Jetton, M. R. 166  
 Jetton, M. A. 362  
 Johar, D. S. 302, 307  
 Johns, C. O. 13  
 Johnson, B. 76  
 Johnson, B. C. 73, 76, 77, 167  
 Johnson, D. W. 374  
 Johnson, S. 368  
 Johnsson, S. 179  
 Johnston, C. H. 295  
 Jolles, G. 58  
 Joly, M. 334  
 Jones, C. M. 404  
 Jones, D. B. 13  
 Jones, H. 153  
 Jones, S. 317  
 Jones, W. A. 54  
 Jones 69  
 Joseph, D. 146  
 Joseph, K. 276, 286, 369, 374,  
 396, 420, 430  
 Josephson, K. 99  
 Jukes, T. H. 122

## K

Kabat, E. A. 128  
 Kalbag, S. S. 260, 264, 266, 269,  
 368  
 Kalnitsky, G. 46  
 Kaneko, T. 311  
 Kaplan, B. G. 112

Kaplan, J. 46  
 Kaplan, N. O. 166, 203  
 Kapur, N. S. 274, 275  
 Kar, A. B. 179, 180  
 Karkun, J. N. 175, 179, 180  
 Kasbekar, D. K. 167  
 Kassell, B. 66  
 Katsh, G. F. 171  
 Katsh, S. 171  
 Kaufman, S. 7  
 Kawishwar, W. K. 128  
 Kech, K. K. 106  
 Kegeles, G. 6, 7  
 Keilin, D. 196  
 Keilin, P. 210  
 Kensler, C. J. 167  
 Keuther 132  
 Khan, N. A. 362  
 Khorana, H. G. 29  
 Khubchandani, P. G. 306  
 Kihara, T. 311  
 Kimble, M. S. 112  
 King, E. J. 231  
 Kinsell, L. W. 139  
 Kirby 69  
 Klainer, S. M. 6  
 Kleiber, M. 122  
 Klein, D. 362  
 Kobataka, Y. 258  
 Koch 423  
 Koch, A. L. 89  
 Koch, F. C. 66  
 Kocholaty, J. R. 128  
 Kocholaty, W. 128  
 Kodicek, E. 123, 193  
 Koeppe, R. E. 414  
 Koerner, J. F. 29  
 Kokatnur, M. 139, 153  
 Kolb, J. J. 438  
 Kolthoff 60  
 Kornberg, A. 123  
 Kosterlitz, H. W. 167  
 Kothawalla, Z. R. 306  
 Kothary, K. 380  
 Kotsevalov, O. 224  
 Kraaij, G. M. 202  
 Krautman, B. 58  
 Krebs, H. A. 411, 414  
 Krecl, W. A. 146  
 Krejci, L. C. 196  
 Krishnamurthi, C. R. 50, 54, 55,  
 78, 203, 306, 318, 325  
 Krishnamurthy, K. 369, 374, 430  
 Krishnan, B. G. 421, 430  
 Krishnaswamy, M. A. 302  
 Kritchevsky, D. 139, 153  
 Kritzman, M. G. 210  
 Kuby, S. A. 82  
 Kuchel, C. C. 328  
 Kulkarni, L. 357, 362  
 Kumar, S. A. 226  
 Kummerow, F. A. 139, 153  
 Kunkel, H. G. 19  
 Kunitz, M. 46  
 Kuppaswamy, S. 264, 274, 284,  
 295, 300, 374, 420  
 Kurien, P. P. 54  
 Kurt Weiss, A. 171  
 Kyte, R. M. 300

## L

Labo, C. 122  
 Lacombe, G. 66  
 La Du, B. N. 202  
 Lahiry, N. L. 296, 300, 312, 317,  
 335  
 Lakshmanan, M. R. 239  
 Lal, G. 435, 438  
 Lal, S. B. 362, 421, 429, 430  
 Landgrebe, T. W. 179, 180  
 Landsteiner, K. 224  
 Lardy, H. A. 82  
 Laszt 123  
 Lavate, W. V. 140, 167  
 Lazarus, M. 247  
 Lea, C. H. 202  
 Leaf, G. 166  
 Lechow, B. 123  
 Ledingham, U. 404  
 Lee, D. D. 139  
 Lee, T. H. 180  
 Lee, J. G. 378, 380  
 Lehman, L. 19  
 Leonard, S. J. 311  
 Leong, K. C. 146, 153  
 Lerner, A. B. 180  
 Levenson, S. M. 404  
 Levin, E. 295, 300  
 Levine, S. Z. 128  
 Levintow, L. 238  
 Levy, A. L. 46, 66  
 Levy, B. B. 202  
 Levy, R. H. 89  
 Lewis, J. M. 112  
 Lew, W. 139  
 Li, C. H. 179, 180  
 Liener, I. E. 76, 378, 380  
 Lillerick, A. A. 362  
 Lillie, R. D. 414  
 Lindgren, F. 153  
 Link, K. P. 128  
 Linton, R. W. 196  
 Lipmann, F. 166, 193, 238  
 Litwack, G. 112, 167  
 Lobay, W. 433  
 Lock, J. A. 179  
 Lockwood, L. B. 311  
 Loewi, G. 193  
 Logan, J. B. 139, 153  
 Long, C. N. H. 167  
 Longnecker, J. B. 352, 354  
 Longwell, B. B. 171  
 Loomis, W. D. 238  
 Loosli, J. K. 112  
 Lopez, Matas, A. 295  
 Lopez, G. C. 404  
 Love, R. H. 166  
 Loveen, M. K. 334  
 Lowher, A. G. 66  
 Lowry, O. H. 29, 82, 99, 106, 123  
 166, 203, 253  
 Luck, J. M. 166  
 Luckey, T. D. 99  
 Luh, B. S. 311  
 Lulla, B. S. 307  
 Luman, F. 295  
 Lund, C. C. 404  
 Lyman, M. M. 153  
 Lyon, T. 153



## M

Ma, T. S. 196, 334  
 Maass, A. R. 122  
 Mabee, D. 334  
 MacDonald, M. R. 89  
 Macdonald, H. A. 410, 413  
 Macfadyen, D. A. 49  
 Macheboeuf, M. 372, 374  
 Magsanik, B. 99  
 Mahadevan, V. 328  
 Mahdihassan, S. 216  
 Maignon, F. 146  
 Main, R. A. 180  
 Malik, D. A. 374  
 Mandel, H. G. 97, 99  
 Mandelstam, J. 82  
 Mann, T. 46, 225  
 Manzini, C. 185  
 Marais, J. S. C. 363  
 March, B. S. 328  
 March, B. E. 300  
 Marfatia, U. M. 133, 146, 147, 166  
 Mariam de Salegai 132  
 Mark, D. D. 166  
 Markham, R. 97, 99, 410  
 Murkley, K. S. 275, 311  
 Marko 69  
 Markovitz, A. 193  
 Marshall, F. H. A. 46, 247  
 Martin, A. J. P. 225, 362, 440  
 Martin, E. A. 430  
 Mason, M. 123  
 Masson, J. G. 237  
 Massey, V. 7  
 Master, R. W. P. 25, 29  
 Matterson, L. D. 146  
 Matthews, R. E. F. 99  
 Maurer, P. H. 36  
 Mathur, M. C. 433  
 May, C. D. 128  
 Mayer, M. 123  
 Mayer Gross, W. 185  
 Mcelroy, L. W. 433  
 McClendon, J. H. 253  
 McDermott, E. E. 54  
 McDonald 410  
 McElvin 247  
 McGuire, G. 112  
 McLaughlon, J. M. 295  
 McMeekin 423  
 McMeekin, T. L. 66  
 Meena Rao, J. 181  
 Mehl, J. W. 24  
 Mehta, C. J. 244  
 Mehta, G. 374, 387, 392, 396, 420  
 Mehta, R. 76, 77, 167  
 Meier, R. 193  
 Meikleham, V. 167  
 Meister, A. 238  
 Mellinkoff, S. M. 185  
 Mendal, L. B. 132, 284  
 Mendel, T. L. B. 289, 374, 380  
 Mendel, T. L. B. 356

Merril, J. F. 301  
 Merritt, J. B. 377, 380  
 Meshyan, G. Y. 171  
 Metcalf, R. L. 112  
 Mehta, V. C. 146  
 Michaud, L. 122  
 Michel, R. 66  
 Michaels, G. D. 139  
 Michael Green, N. 7  
 Michelson, C. 238  
 Milb, R. C. 380  
 Milchel, C. A. 362  
 Micks, D. W. 440  
 Miller, D. S. 295, 301  
 Miller, E. R. 378, 380  
 Miller, L. L. 166  
 Mills, R. C. 238  
 Mingioli, E. S. 78, 82, 106  
 Minor, L. J. 311  
 Mirsky 69  
 Mirsky, A. E. 334  
 Misra, S. B. 196  
 Misra, R. P. 414  
 Mistry, S. P. 76  
 Mitbander, B. V. 166  
 Mitchell, G. M. 180  
 Mitchell, H. H. 146, 284, 345, 352, 374  
 Mitra, B. N. 196  
 Mitra, K. 353  
 Mitra, S. 197  
 Moffat, N. M. 146  
 Mohanty, S. B. 316  
 Moldave, M. 77  
 Monod, J. 82, 106  
 Monson, W. S. 76, 167  
 Mookerjea, S. 114  
 Moore, P. R. 112  
 Moore, T. 112  
 More, S. 19  
 Moorefield, H. H. 439, 440  
 Moorjani, M. N. 274, 296, 300, 301, 430  
 Morgan, A. F. 123, 334  
 Morris, M. P. 378, 380  
 Morris, C. J. O. R. 179  
 Morrison, A. B. 290, 295  
 Morrison, R. I. 253  
 Mosher 69  
 Morton, R. A. 112, 363, 368  
 Moyer, A. W. 139, 153  
 Mukerji, B. 175, 179, 180  
 Mukerjee, S. 196  
 Mukherjee, K. L. 414  
 Mukherjee, R. 433  
 Mulgaonkar, A. G. 76, 139, 140, 167  
 Munro, H. M. 146  
 Munro, H. N. 146, 185  
 Muntwyler, E. 166  
 Murphy, E. A. 247, 363, 368  
 Murray, A. 179  
 Murray, H. C. 334, 374  
 Murray, T. K. 341  
 Murthy, V. S. 76

## N

Nadkarni, G. B. 166  
 Naganna, B. 231  
 Nagchaudhuri, J. 405  
 Nagy, L. 171  
 Nandor Porges 29  
 Nanjundaswamy, A. M. 329  
 Narasimha Murty, P. V. 61  
 Narasinga Rao, M. S. 1, 7  
 Narayana, K. 260  
 Narayana, M. N. 260  
 Narayana, N. 54, 362, 433  
 Narayanan, K. M. 274, 275  
 Narayana Rao, M. 54, 276, 286, 289, 368, 369, 374, 393, 396, 420, 430  
 Nath, M. C. 433  
 Nath, N. 139, 153, 434  
 Nath, R. 13, 360, 362  
 Nayar, J. K. 439, 440  
 Neidhardt, F. 106  
 Neish, J. P. 414  
 Neuberger, A. 166  
 Neurath, H. 7, 46  
 Ney, P. W. 317  
 Ni, S. M. 311  
 Nichol, C. A. 166  
 Nicholls, D. 171  
 Nickerson, J. T. R. 328  
 Nilsson, I. M. 24  
 Nisman, B. 210  
 Nitya Anand 100  
 Niyogi, S. P. 54, 434, 362  
 Noble, N. L. 171, 193  
 Northover, B. J. 193  
 Northrop, J. H. 238  
 Norton, H. W. 122  
 Novellie, L. 317  
 Nunez, J. 66  
 Nussbawmer, T. 24

## O

Obata, Y. 311  
 Ochoa, S. 123  
 O'Doherty, K. 146  
 Oginsky, E. 76  
 Oginsky, E. L. 166  
 Ogston, A. G. 7  
 Okey, R. 153  
 Olden, J. H. 300  
 Onaga, D. M. 311  
 Ontko, J. A. 153  
 Osborne, T. B. 13, 284, 289, 356, 355, 371, 374, 378, 380  
 Oser, L. B. 430  
 Osher, P. 171  
 Ozulak, J. 306



## P

Pace, J. 54  
 Padilla, A. M. 123  
 Pagan, C. 378, 380  
 Pal, S. 67  
 Palade, G. E. 166  
 Pangborn, M. 202  
 Pant, N. C. 439, 440  
 Pant, K. D. 194  
 Papageorge, E. 171  
 Pappenheimer, A. M. 82  
 Pardee, A. B. 89, 99  
 Parpia, H. A. B. 274  
 Pashkina, T. S. 123  
 Pasternak, C. A. 193  
 Patwardhan, V. N. 112, 289, 387  
 Pattabiraman, T. N. 187  
 Patton, A. R. 76, 167  
 Paul, W. 146  
 Pauling, L. 19  
 Payne, W. W. 185  
 Pedersen 6  
 Pederson, D. J. 112  
 Peek, N. F. 153  
 Peretianu, J. K. 112  
 Perkins, T. A. 301  
 Perkinson, J. D. 128  
 Perlzweig, W. A. 122, 167  
 Perur, N. G. 248  
 Peterson, D. W. 153  
 Peterson, E. A. 29  
 Pethybridge, S. I. 433  
 Petric, A. H. K. 237  
 Phansalkar, S. V. 112, 345, 346, 348, 354, 386, 387  
 Phillips, P. H. 153, 166  
 Phillis, E. 237  
 Pierard, A. 210  
 Pieroni, P. F. 179  
 Pillai, V. K. 300  
 Pillai, N. C. 19  
 Plazin, A. 123  
 Pollack, H. 341  
 Pollister 69  
 Pollister, A. W. 171  
 Pollitzer, R. 196  
 Poo, L. J. 139  
 Popenoe, E. A. 24  
 Porath, J. O. 179  
 Porath, J. 180  
 Porter, R. R. 46  
 Porter, T. 112  
 Potter, L. M. 146  
 Potter, V. R. 166  
 Potjieter, M. 429, 430  
 Poulik, M. D. 29  
 Pradhan, D. S. 83  
 Pradhan, V. G. 212, 216  
 Price, W. W. 306  
 Prins, H. K. 19  
 Pritchard, H. 300  
 Proctor, B. E. 300, 328  
 Pruthi, J. S. 435, 438  
 Purvis, J. L. 180  
 Purushowtham Rao, G. 389, 392  
 Putman 69

## Q

Quenouille, M. H. 167

## R

Radhakrishnamurthy, R. 166  
 Radhakrishnan, A. N. 66  
 Radha Karnad 415  
 Raghupathy, E. 61  
 Raisz, L. G. 247  
 Rajagopal, K. 429, 430  
 Rajagopalan, K. V. 317  
 Rajagopalan, R. 276, 374, 430  
 Rajasekharan, N. 274, 275  
 Ramachandra, B. S. 260  
 Ramachandran, M. 346, 353, 354, 387  
 Ramakrishnan, T. 204, 374  
 Ramalingaswamy, V. 289, 387  
 Raman, A. 231  
 Ramanathan, N. K. 387  
 Ramanna, B. R. 260  
 Rama Rao, G. 274, 284, 374, 420  
 Rama Rao, P. B. 374  
 Ramasarma, G. B. 123  
 Rand, N. T. 123, 139, 153, 239  
 Randall, R. J. 29, 82, 99, 106, 203, 253  
 Randoin, L. 139  
 Ranganna, S. 329, 334  
 Rao, G. 275  
 Rao, G. J. S. 15, 16  
 Rao, M. N. 274  
 Rao, M. V. L. 129, 181, 264, 438  
 Rao, N. A. N. 204, 225, 438  
 Rao, Shanta S. 218, 224, 225  
 Rao, S. S. 25, 29, 31, 225  
 Rao, T. 438  
 Rao, T. B. 133, 147  
 Rao, V. S. 49  
 Rastogi, M. K. 318, 325  
 Ray 129, 132  
 Ray, A. 356  
 Razzell, W. E. 29  
 Reay, G. A. 328  
 Recheigl, M. (Jr.) 112  
 Reddy, S. K. (Miss) 275, 284, 430  
 Rege, D. V. 83, 90, 166, 167  
 Reid, E. 179  
 Resnick 69  
 Rhoads, C. P. 167  
 Rhodes, D. N. 202  
 Rice, E. E. 334  
 Richert, D. A. 167  
 Richter, C. P. 146  
 Ris, H. 166  
 Rittenberg, D. 123  
 Robbler, A. R. 146  
 Robinson, R. W. 231  
 Robertson, J. J. 89  
 Roberts, S. 123  
 Robscheit-Robbins, F. S. 122  
 Roche, J. 61, 66, 180  
 Rodriguez, L. D. 433  
 Roe 132  
 Rogers, C. G. 295  
 Rogers, S. N. 301

Roger, W. L. 46  
 Rohatgi, K. S. 128  
 Roichaudhuri, B. M. 168  
 Romoser, G. L. 146, 153  
 Roos, P. 180  
 Rose, W. C. 345, 353, 368, 376, 380  
 Rosebrough, H. J. 29, 82  
 Rosebrough, N. J. 29, 82, 99, 106, 203, 253  
 Rosenberg, H. R. 325, 374  
 Roseman, S. 193  
 Rosen, G. D. 368  
 Rosenthal, H. L. 146  
 Rossiter, R. J. 123, 171  
 Roth, H. 106  
 Roveosi, L. 122  
 Rowlands, I. W. 202  
 Roy, A. B. 316  
 Roy Chaudhuri, D. 326  
 Roy, D. K. 258  
 Roy, H. 128  
 Roy, S. K. 179  
 Roe 132  
 Roy, S. C. 362  
 Ruata, C. Q. 202  
 Rubin, L. 166  
 Rudra, M. N. 362  
 Ryser, H. 122

## S

Sadaphal, M. N. 433  
 Sadhu, D. P. 172, 174  
 Sadri, K. K. 218, 224, 225  
 Sahli-Adams 397  
 Sakamura, Y. 311  
 Salem, W. J. 6  
 Salisbury, P. F. 247  
 Salmon, R. J. 128  
 Salmon, W. D. 167  
 Salt, H. B. 410, 414  
 Samuels, L. T. 167  
 Sankaran, A. N. 275, 284, 289, 374, 420, 430  
 Sarett, H. P. 122, 167  
 Sarkar, N. K. 414  
 Sarma, P. S. 61, 166, 317  
 Satyanarayana, N. S. 350, 354  
 Sauberlich, H. E. 166  
 Saxena, K. C. 325  
 Sayers, G. 171  
 Sayers, M. A. 171  
 Schachman, H. K. 7, 171  
 Schaefer, A. E. 167  
 Schaefer, H. C. 368  
 Schaldler, R. W. 325  
 Schiller, S. 193  
 Schmid, K. 24  
 Schmidt, E. G. S. 247  
 Schnakenberg, G. 295  
 Schneider, D. C. 106  
 Schneider, W. C. 58, 89, 91, 99, 166  
 Schoenheimer, R. 139  
 Schreiber, M. 146, 153  
 Schuler, W. 193



- |                         |                         |                    |   |                    |   |
|-------------------------|-------------------------|--------------------|---|--------------------|---|
| Schulz, D. M.           | 36                      | Smith, A. K.       | 311   | Subba Rao, V.      | 47  |
| Schultz, M. O.          | 76, 375, 380            | Smith, E. P.       | 362   | Subba Rao, Y.      | 193, 203, 231, 238  |
| Schwartz                | 132                     | Smith              | 132   | Subrahmanyam, V.   | 54, 260, 264, 269, 270, 274, 275, 284, 286, 289, 295, 300, 306, 307, 368, 369, 374, 396, 415, 418, 420, 424, 429, 430 |
| Schwarz, K.             | 325                     | Smith, C. L.       | 46  | Subramanian, N.    | 54, 260, 264, 269, 363, 368, 393  |
| Schweigert, B. S.       | 76, 375, 166            | Smith, M. J.       | 406, 414  | Subramanyan        | 424   |
| Schwemmin, D.           | 89                      | Smith, M. S.       | 139   | Sugarman, G. I.    | 99  |
| Schwert, G. W.          | 7                       | Smith, P.          | 139   | Sukanya, N. K.     | 232   |
| Scott, H. M.            | 139, 153                | Smith, V. R.       | 146   | Sullivan, W. R.    | 128   |
| Scrimshaw, N. S.        | 289, 369, 371, 374, 396 | Smith              | 406   | Summerson, H. W.   | 430   |
| Seagram, H. L.          | 328                     | Smithies, O.       | 25, 29  | Sulman, F. G.      | 179   |
| Seal, S. C.             | 196                     | Smoletis, A. M.    | 58  | Summer, J. B.      | 29, 196   |
| Sealock, R. R.          | 128                     | Smuts, D. B.       | 363, 368  | Sunde, M. L.       | 146, 153  |
| Sebrell, W. H.          | 123                     | Smyth, J. M.       | 24  | Supplee, W. C.     | 146   |
| Seifter, S.             | 166                     | Snow, J. M.        | 328   | Sur, G.            | 430   |
| Sekhara Varma, T. N.    | 239                     | Snyder, F.         | 203   | Sure, B.           | 122, 139, 295, 300, 374   |
| Seligman                | 171                     | Sobel, A. E.       | 123   | Susheela, R.       | 435, 438  |
| Sellers, E. A.          | 146                     | Sober, H. A.       | 29, 122   | Suzuki, S.         | 193   |
| Selye, H.               | 171                     | Sohonie, K.        | 300, 362, 375, 380                                      | Svedberg, T.       | 1, 6  |
| Sen, D. P.              | 312, 317                | Som, J. M.         | 107, 112, 362, 434                                      | Swaminathan, M.    | 54, 76, 264, 274, 275, 276, 284, 286, 289, 345, 346, 368, 353, 420, 430, 369, 374, 396                                |
| Sen, P. B.              | 168                     | Someswara Rao, K.  | 387   | Swaminathan, M. C. | 387   |
| Senecal, J.             | 185, 295                | Southcott, B. A.   | 300, 317  | Swan, J. M.        | 54, 368, 396  |
| Sen Gupta, A.           | 203                     | Speck, R. J.       | 106   | Swanson, P. R.     | 146   |
| Sephton, H. H.          | 317                     | Spector, H.        | 122, 146  | Sweemy, L.         | 196   |
| Serrain, C.             | 19                      | Sperry, W. M.      | 139   | Swift, R. W.       | 146   |
| Sevag                   | 69                      | Speyer, J. F.      | 167   | Szafranski, P.     | 77  |
| Shah, S. L.             | 31                      | Spiegelman, S.     | 99  |                    |   |
| Sharma, S. K.           | 194                     | Spitzer, E. H.     | 123   |                    |   |
| Shaw, C. R.             | 216                     | Sreenivasan, A.    | 76, 83, 90, 123, 133, 140, 146, 147, 154, 166, 167, 260 |                    |   |
| Sheila, H.              | 301                     |                    |   |                    |   |
| Shemin, D.              | 123                     | Srikantia, S. G.   | 374, 381, 387, 392, 396, 420                            |                    |   |
| Shenstone, F. S.        | 316                     | Srinivasan, M.     | 129, 181, 185, 295, 300, 362, 374                       |                    |   |
| Shepherd                | 180                     |                    |   |                    |   |
| Sherman                 | 132                     | Srinivasan, P. R.  | 346, 354, 362   |                    |   |
| Sherman, H. C.          | 146                     | Sripathi, C. A.    | 231   |                    |   |
| Sheth, A. R.            | 218, 225                | Sripathy, N. V.    | 317, 335  |                    |   |
| Shils, M. E.            | 350, 354                | Sriramachari, S.   | 354, 386, 387   |                    |   |
| Shonk, C. E.            | 166                     | Stacey             | 69  |                    |   |
| Shorr, E.               | 139, 166                | Stachiewicz, E.    | 106   |                    |   |
| Shrimpton, D. H.        | 300                     | Stanier, M. W.     | 139   |                    |   |
| Shrivastava, D. L.      | 194, 196                | Stare, F. J.       | 153, 404  |                    |   |
| Shukla, O. P.           | 55, 57                  | Stauffer, J. F.    | 89, 139, 166, 174                                       |                    |   |
| Shuttleworth, R. S.     | 317                     | Stavitsky, A. B.   | 225   |                    |   |
| Sibbald, I. R.          | 146, 153                | Steinrauf, I. K.   | 46  |                    |   |
| Siddappa, G. S.         | 329, 334                | Steele, J. M.      | 202   |                    |   |
| Simkin, J. L.           | 77, 167                 | Steele, B. F.      | 216   |                    |   |
| Simpkiss, M. J.         | 185                     | Stein, W. H.       | 19  |                    |   |
| Singer, H. A.           | 19                      | Steiner, R. F.     | 7   |                    |   |
| Singer, M. O.           | 167                     | Stekol, J. A.      | 139   |                    |   |
| Singh, C.               | 325                     | Stenger            | 60  |                    |   |
| Singh, H. D.            | 318, 128                | Stephens, N.       | 203   |                    |   |
| Singh, D. K.            | 434                     | Steward, F. C.     | 237   |                    |   |
| Sing, Rattan            | 433                     | Stewart, M. B.     | 350, 354  |                    |   |
| Singson, E. P.          | 146                     | Stinson, L. D.     | 146   |                    |   |
| Sinisterra, L.          | 153                     | Stitch, W.         | 123   |                    |   |
| Sinsheimer, R. L.       | 29                      | Stoll, R. D.       | 202   |                    |   |
| Sirex, F. M.            | 325                     | Stoppani, A. O. M. | 179   |                    |   |
| Sirsi, M.               | 204                     | Straub, F. E.      | 325   |                    |   |
| Sivaramakrishnan, V. M. | 239, 243                | Straub, G. J.      | 166   |                    |   |
| Skeggs, H. R.           | 166                     | Straumfjord        | 132   |                    |   |
| Skellenger, M.          | 216                     | Strauss, M. B.     | 247   |                    |   |
| Skipin, A. I.           | 269                     | Strecker, H. J.    | 210   |                    |   |
| Skreb, Y.               | 89                      | Streibich, M. J.   | 58  |                    |   |
| Slack, E. B.            | 289                     | Strength, D. R.    | 167   |                    |   |
| Slack, H. G. B.         | 193                     | Strisower, B.      | 153   |                    |   |
| Slater, E. C.           | 112                     | Strominger, J. L.  | 193   |                    |   |
| Slater                  | 132                     | Stumpf, P. K.      | 238   |                    |   |
| Smadel, J. E.           | 106                     |                    |   |                    |   |

## T

- |                     |               |
|---------------------|---------------|
| Tabor, H.           | 123           |
| Tamura, A.          | 258           |
| Tamura, E.          | 258           |
| Tan, W.             | 66            |
| Tapley, D. F.       | 36            |
| Tappen, N. C.       | 19            |
| Tarr, H. L. A.      | 295, 300, 317 |
| Tasker, P. K.       | 374, 430      |
| Tauber, H.          | 414           |
| Taussky, H. H.      | 139, 166      |
| Tawde, Saroj        | 8, 13         |
| Tayalu, F.          | 374           |
| Taylor, G.          | 341           |
| Taylor, H. C. (Jr.) | 167           |
| Taylor, K. W.       | 406, 407, 414 |
| Teakle, L. T. H.    | 433           |
| Terrill, S. W.      | 122           |
| Thacker, E. J.      | 146           |
| Thing               | 179           |
| Thomas, Y.          | 89            |
| Thompson, R. E.     | 179           |
| Thomson, W. S. T.   | 146, 185      |
| Thorne, G. N.       | 433           |
| Thornton, N.        | 185           |
| Thorpe, R. M.       | 36            |
| Tipnis, H. P.       | 166           |
| Tiselius, A.        | 7, 128        |
| Toennies, G.        | 438           |
| Tokoyama, S.        | 300           |
| Tombs, M. P.        | 15, 19        |



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# SUBJECT INDEX

## A

- Active sulphate synthesising system in granulation tissue, 188, 189
- Actomyosin, denaturation of, in fish muscle, 326
- Adrenocorticotrophic hormone, 175
  - structural and functional relation to MSH, 175-179
  - amino acid sequence of, 178
- Alanine dehydrogenase in *Mycobacteria*, 204
  - purification of, 205-206
  - characteristics of, 206-207
  - inhibition of by iodoacetate and PCMB, 208
  - mechanism of action of, 209
- Alkaline phosphatase, 226
  - in plants, 226
  - from green gram, 226
  - properties of, 227-229
  - effect of ferric ion on, 228
  - ferric ion requiring, in plants, 230
- Archibald method, molecular weight determination by, 1
  - ultracentrifuge pattern in, 3
- Aspergillus oryzae*, 309

## B

- Bacillus subtilis*, 321
- Banana and mango custard blends, 329
- Barbus carnaticus*, 314
- Bengal gram, annual production of, 276
  - high and low protein varieties, 357
- Bengal gram protein, 357
  - nitrogen distribution in, 357
  - nutritional assessment of, 357
  - fractionation of, 360
  - NPN analysis of, 361
  - protein efficiency ratio of, 367
- Bovril, 310
- Buffalo hemoglobin, heterogeneity of, 15-19
  - alkali denaturation of, 15
  - salting-out studies on, 15
  - agar-electrophoresis of, 16-17
  - ion-exchange chromatography of, 17-18

## C

- Cajanus cajan*, 355
  - proteins of, 8
- Casein, 367, 393
  - effect of feeding of, on body weight and liver constituents of protein-depleted rats, 165
  - protein efficiency ratio of, 367
- Catalase synthesis, in yeast, relation to RNA metabolism, 90-99
  - effect of aeration on induction of, 91
  - effect of composition of culture medium on, 92
  - inhibition by azaguanine, bromouracil and nitrouracil, 92, 94
  - influence of phosphate starvation on, 92
  - relation to RNA synthesis, 92
  - incorporation of glycine into protein and RNA during, 93
  - effect of RNA, amino acids and casein hydrolysate on, 94-95

- effect of cystine and EDTA, 95
- reversal of cystine inhibition by  $Fe^{+++}$ , 96
- during aerobic growth, 97

- Cereals, 347
  - PER of proteins of 347, 348
  - protein content of, 430
- Cetrimide, 68
- Cheese, Cheddar, preparation of, with *Ficus* enzyme, 302
  - processed, 306
  - composition of, 304
  - changes during ripening of, 305
- Chicken essence, preparation of, 335, 339
  - composition of, 341
- Chloramphenicol effect on *E. coli* cells along with streptomycin, 105
- Chondroitin sulphate, incorporation of  $S^{35}$  into, 190
- Chymotrypsin, molecular weight vs. concentration, 3
  - sedimentation velocity pattern of, 4
- Cicer arietinum*, 415
- Clostridium botulinum*, 314
- Clupea longiceps*, 268
- Cobalt, 60
  - distribution between blood cells, serum and serum proteins, 242
  - distribution among serum proteins, 243
- Cobalt-60 transport, 239
  - by blood proteins, 239
  - influence of chelating agents on, 239
- Cobra venom proteins, separation using ion-exchangers, 25
  - enzyme activity of, 27
  - fractionation of, using IRC-50, 27
  - using DEAE cellulose, 28
- Composite protein food, nutritional deficiency score as affected by, 429
  - blood picture as affected by, 429
  - blood proteins as affected by, 429
- Composite protein food, 421
  - in school lunch programme, 421
  - clinical response to, 424
  - weight of school children as affected by, 425
- Composite protein food, height of school children as affected by, 426
  - Haemoglobin as affected by, 427
  - blood cell count as affected by, 428
  - hip width as affected by, 429
- Creatinine, in meat extract, 335

## D

- DANB derivatives, 406
  - of pyruvic acid, 406
  - of alpha-ketoglutaric acid, 406
  - extraction of, 407
  - estimation of, 407
  - recovery of, 409
  - electrophoretic separation of, 413
- Dietary protein and B-vitamins, relation to regeneration of tissue constituents in protein depleted rats, 154-167
- Dietary protein quality and fat levels, effect on



- lipid composition and distribution in blood and liver, 147-153
- Dietary protein quality, influence on body weight and liver constituents during protein-starvation and refeeding, 163
- Dietary protein, quality and level, influence on body composition, 133-139
  - effect on growth and food intake, 134
    - on carcass composition, 135
    - on tissue nitrogen lipids and phospholipids, 136
    - on organ: body weight ratios, 136
    - on blood picture, 137
    - on plasma nitrogen, lipids and phospholipids, 137
    - on liver and plasma cholesterol, 138
    - on lipid distribution in  $\alpha$ - and  $\beta$ -lipoproteins of plasma, 138
- Difco peptone, nitrogen distribution in, 314
- Diphtheria antitoxic sera, effect of heat on, 33, 34
- DNA and RNA from mammalian tissues, 67
  - separation of proteins from, a simple procedure for, 67-68
  - composition of, from liver, 68
- Double bean, 375
  - nutritive value of, 375
  - biological studies on, 375
  - amino acid composition of, 376
  - antigrowth factor in, 378
- Double bean diet, 377
- Double bean protein isolate, 378
  - growth promoting value of, 379

## E

- Effect of acids on proteins, 37
- Elasmobranchs, 316
- E. coli*, synthesis of cellular constituents by, 78, 79
  - induced synthesis of galactosidase and permease in, effect of  $B_{12}$  on, 81

## F

- FAO food balance sheets, 270
- FAO protein reference pattern, 369
- Ficus carica* L., 302
- Fish bacto-peptones, 312
  - enriched products, 298
- Fish flour, 290
  - nutritive value of, 290, 292
  - South African, 291
  - Canadian, 292
  - effect on nutritional value of white bread, 293
  - Dabsch process for, 290, 296
  - Vogel process for, 290, 296
  - Viobin process for, 290, 296
  - Japanese process for, 296
  - Russian process for, 296
  - South African process for, 290, 291, 296
- Fish macaroni, cooking quality of, 299
- Fish, products of protein hydrolysis from, 312
  - hydrolysates, 312
  - utilization of total production in India, 315
- Fish protein, biological value of, 292
- Fish sauce, 312
- Fish tissue, protein denaturation in, 326
  - analysis of different protein fractions in, 327
- Formaldehyde, reaction with peptone, 47-49
  - determination of bound, 47

## G

- Glucose tolerance, influence of proteins and amino acids on, in normal and diabetic subjects, 181-185
  - effect of leucine, on, 183, 184
- Glutamine synthesis, 232
  - inhibitor of, in *Pongamia* galls, 232
  - solubilisation of the inhibitor of, 233
  - properties of the inhibitor of, 234
  - action of the inhibitor of, 235
- Glutamotransferase from peas, 232
  - from sheep brain, 232
  - from *Cucurbita pepo*, 232
- Gnorimoschema operculella*, Zell, 439
- Granulation tissue, enzymes in, 190
- Grass proteins, 433
- Groundnut, 261
  - integrated processing of, 261
  - modified process for, 263
- Groundnut, study of operations in the integrated process, for 266
  - separation of protein from, 267
- Groundnut protein, 364, 265, 387
  - digestibility coefficient of, 364
  - biological value of, 364
  - protein efficiency ratio of, 365
  - nitrogen retention value of, 365

## H

- Haddocks, 327
- Hansenula subpelliculosa*, 309
- Haemolysate of buffalo blood, 13
- Haemopoiesis, 397
- Heart digests and infusions, 214
  - growth response of bacteria to, 214
- Heart extracts, 212
  - microbiological evaluation of, 212-217
    - amino acid composition of, comparison with whole heart and meat, 212
  - microbial growth promotion, for pharmacological assay of, 217
  - growth response of *C. septicum* to, 215
- Horse serum, effect of heat on proteins in, 31, 33
  - electrophoresis of, 32
  - effect of glutathione and iodoacetate on protein interactions in, 34
  - immuno-electrophoresis of, 32
  - interaction of proteins in, 31
- Hexametaphosphate, for isolating basic proteins, 55
  - isolation of lysozyme by, 56
  - precipitation of lytic enzymes from rabbit spleen by, 57
  - preparation of basic proteins from rabbit liver nuclei with, 57

## I

- Iodine, estimation in protein complexes, simple method for, 59-60
- Iodothyris, 61-66
  - isolation from cattle thyroglobulin, 62
  - nitrogen, iodine and hexosamine content of, 63
  - amino acid composition of, 64
  - from hog and sheep thyroglobulins, 65
  - end group analysis of, 63
- Iron chlorosis, 248
  - effect of, on protein fractions of corn leaf, 248-253



## K

- Krebs cycle oxidation, in rat, toad and pigeon  
exposed to cold, 173  
Kwashiorkor, 385, 389

## L

- Labio rohita*, 326  
*Lactobacillus arabinosus*, 321, 355  
*Lactobacillus casei*, 321  
*Lactobacillus delbrueckii*, 309, 321  
*Lactobacillus fermenti*, 321  
*Lactobacillus leichmanii*, 348  
Lathyrism, 378  
*Lathyrus sativus*, 354, 378  
Leaf protein, technology of, 257  
extractants for, 257  
refining of, 257  
supplement to cereal diets, 258  
acceptability of, 259  
residue after extraction of, uses for, 258, 259  
Leucine, effect on glucose tolerance and insulin  
action in normal subjects, 183-184  
effect on blood sugar and plasma insulin activity  
in infantile hypoglycemia, 186  
Lipoproteins, 410, 411  
Livers, 351  
vitamin B<sub>12</sub> activity of, 351  
cirrhosis of, 399  
*Luconostoc mesenteroides*, 355  
Legume protein, 433  
*Lens esculenta*, 355  
Lysozyme, isolation and purification with hexame-  
taphosphate, 57, 58

## M

- Mackerel, 314  
Mango custard blend and powder, 330, 331  
Marmite, 310  
Malt food, balanced, 283  
supplementary value of, 283  
Meat extract, preparation of, 335  
analysis of, 336, 337  
Meat, minced, analysis of, 335  
Meat soup, cubes, 337  
mix, analysis of, 339  
Melanocyte stimulating hormone (MSH), human,  
hog, and ox, amino acid sequence in, 178  
Microbial process, for predigested protein foods, 307  
'Miso', 307  
Mucopolysaccharides, metabolism in connective  
tissue, 187  
sex differences in synthesis of, 191, 193  
 $\alpha_1$ -muco protein from sheep plasma, 20  
isolation of, 20  
agar-electrophoretic pattern of, 22  
reactions of, 23  
hexosamine content of, 24  
Multipurpose food, American, 278  
Multipurpose food, Indian, 276-277  
flow-sheet for manufacture of, 277  
overall nutritional value of, 278, 280  
supplementary value to poor Indian diets, 279

- biological value of, 280  
formula C for, 283  
in the treatment of Kwashiorkor, 285  
MPF, 390, 392, 415  
clinical response to, 391  
haemoglobin and serum protein increases  
due to, 391  
a low cost protein supplement, 415  
feeding trials with, 415, 416  
in school lunch programmes and homes, 417  
*M-lacticola* alanine dehydrogenase from, 208  
*M-tuberculosis*, H<sup>37</sup>RV, alanine dehydrogenase from,  
208

## N

- Naja naja*, proteins of the venom of, 25  
Nitrogen retention and growth at varying dietary  
levels of protein, fat and protein: calorie ratio, 140  
influence of pantothenic acid, folic acid, vitamin  
B<sub>12</sub>, choline and methionine, 142  
influence of B<sub>12</sub> and folic acid, 143  
'Nutro' foods, protein rich, 270-275  
biscuits, 272-271, 418  
breakfast food, 270-274  
macaroni, 270-273  
composition of, 270  
Nutritional oedema syndrome, 395, 418

## O

- Oil sardine, 297, 316  
products from, 297  
composition of, 298  
Oil seeds, annual production of, 276  
Orphanage diet, 422  
composition of, 422  
nutritive value of, 422

## P

- Passiflora edulis*, 435  
Passion fruit, 435  
nitrogenous substances in, 435  
chemical composition of, 436  
fractionation of nitrogen in, 437  
free amino acid distribution in, 437  
Pasty products, protein rich, composition of, 310  
Pepsin, effect of TCA on, 44  
Permeases, 81, 82  
pH5-enzymes, 74-76  
incorporation of B<sub>12</sub> into, 75  
*Phaseolus*, 355  
*mungo*, 355  
*radiatus*, 355  
*Phaseolus radiatus*,  $\beta$ -glycerophosphatase of, 226  
Pigeon pancreas, synthesis of amylase by, 321-322  
*Pisum sativum*, 355  
Plasma proteins, of rabbit blood, solubility after  
TCA precipitation, 40  
fractionation of, 41, 42, 43  
Plasma proteins, 397-399  
fractionation of, 397  
regeneration of, 398  
*Pongamia glabra*, inhibitor of glutamine synthesis  
in the galls of, 232



- Potato moth larvae, amino acid composition of, 439  
 nutritive value of, 439
- Precooked balanced protein food, 286  
 composition of, 287  
 flow-sheet for manufacture of, 286  
 cost of, 288  
 nutritive value of, 289  
 shelf-life of, 289  
 consumer acceptability of, 289
- Protein biosynthesis, role of vitamin B<sub>12</sub>, 73-77
- α-protein, effect of refeeding of, on body weight and liver constituents of protein-fasted rats, 165
- Protein foods, predigested, 307  
 processed, based on oilseed meals, 276  
 flow sheet for, 277-280  
 nutritive value of, 278  
 composition of, 278  
 for weaned infants, 286  
 from fish, 296
- Protein blends, 369  
 nutritive value of, 369  
 PER of, 371, 372  
 nutritive value of, 371, 372, 373  
 amino acid composition of, 394
- Protein fasting, effect on body weight and liver constituents, 156
- Protein hydrolysates, from oil cakes and distillery sludge, 319  
 proximate composition of, 319  
 amino acid content of, 320  
 nutritive value of, 320  
 growth promoting value for microorganisms, 321
- Protein hydrolysates, from fish, 312-316
- Proteolysed fish-liver, 310
- Protein isolates, from groundnut, 260  
 process for, 260-261  
 analysis of, 264
- Protein hydrolysate, 397  
 haemopoiesis in relation to, 397  
 plasma protein regeneration due to, 398  
 haematological response to, 399
- Protein malnutrition, 380, 390, 393  
 biochemical studies on, 405
- Protein—protein interactions, application of Archibald method for study of, 7  
 in horse serum, 31
- Protein utilization, in the growing rat, 140  
 effect of B-vitamins, calories and fat on, 140-146
- Pulse proteins, 355  
 biological value of, 355  
 amino acid composition of, 355, 356, 432  
 supplementation with vitamin B<sub>12</sub>, 433  
*in vitro* digestion of, 433
- R**
- Rastrelliger canagurta*, 314
- Red gram globulins, fractionation of, 8  
 electrophoretic analysis of, 9-13
- Rennet, animal and vegetable, 302
- Riboflavin, influence on nitrogen metabolism and hematopoiesis, 114  
 deficiency in the rat, Hb and RBC content in, 116  
 in hemopoiesis, 114  
 influence of, on hemoglobin regeneration, 117  
 deficiency and cobalt polycythemia, 117  
 influence on liver and blood protein regeneration, 118
- influence on hemoglobin, plasma proteins and liver and kidney composition, 129  
 relation to adrenal cortex, 122
- Rice proteins, 356  
 amino acid composition of, 356
- S**
- Saccharomyces cerevisiae*, 83  
 interdependence of RNA and protein synthesis in, 83  
 effect of phosphate starvation on growth and protein and RNA increments, 85  
 inhibition of protein synthesis in, by p-fluorophenylalanine, 85  
 aminopterin inhibition of RNA and protein synthesis in, 86  
 protein and RNA synthesis in glycine adapted cells of, 87  
 effect of phosphate starvation on viability of, 89
- Scurvy, protein metabolism in, 124-128
- Seminal antigens, 219  
 antibodies to, 219, 221
- Seminal plasma proteins, fractionation of TCA precipitated, 38  
 amino acid composition of, 39
- Serum albumin, effect of heat on, 33
- Serum cholesterol, 388
- Sesame proteins, 367  
 protein efficiency ratio of, 367
- Skim milk, 367, 372, 373, 385, 389, 393  
 protein efficiency ratio of, 367
- Skipin process, 261
- Soya sauce, preparation of, 307, 308  
 composition of, 309  
 large scale preparation of, 309
- Spermatozoa, 218  
 human, antigenicity of, 218  
 antigens of, 219, 223  
 nature of antigens of, 220  
 iso- and auto-antibodies, to, detection of, 223  
 presence of antigens of, in spermatozoal extracts, 222  
 free amino acids and peptides in, 222
- Starch tolerance, effect of casein on, 186
- Strained baby foods, effect of processing on solubility of proteins in, 329  
 proteins in, 330
- Strained pea and field bean pulps, 329, 331
- Streptococcus faecalis*, 355
- Streptococcus hemolyticus*, 314
- Streptomyces*, 321
- Streptomycin, effect on protein synthesis in *E. coli*, 100  
 effect on RNA, DNA and protein synthesis in *E. coli*, 102  
 effect on incorporation of arginine into cellular protein in *E. coli*, 102  
 effect on incorporation of guanine into RNA in *E. coli*, 103  
 effect on β-galactosidase and protein synthesis in *E. coli*, 104  
 effect on RNA, DNA and protein content of *E. coli* cells, 105
- Sulphydryl groups, role in protein interactions in sera, 35



- Suprarenals, protein pattern in cytoplasm of, in cold stressed rats, 168-171  
 cytoplasmic and total protein content of, in cold stressed rats, 170  
 Swan's reagent, comparison with alkali for protein extraction, 50, 52  
 extraction of protein from plant materials with, 51  
 degradation of proteins by, 54

## T

- Tapioca macaroni products, 270  
 Transamination, capacity on cold exposure in animals, 172-174  
 Transamidase in granulation tissue, 189  
 Trichloroacetic acid, effect on seminal plasma proteins, 37  
 effect on blood plasma proteins, 39  
 effect on crystalline enzymes, 44  
 Trypsin, effect of TCA on, 44

## U

- UDPG-pyrophosphorylase and dehydrogenase, 188  
 Uremia, 244  
 protein metabolism in, 244  
 free amino acids in uremic plasma, 245

## V

- Vegetable protein foods, 289, 381, 385  
 clinical trials with, 381  
 nutritive value of, 382  
 preparation of, 382  
 acceptability of, 383  
 clinical response to, 382, 384  
 serum calcium changes due to, 384  
 serum albumin regeneration due to, 384  
 Vegetable protein isolates, 363, 393  
 advantages of, 260  
 nutritional evaluation of, 363  
 formulations of, 363  
 protein efficiency ratio of, 367  
 amino acid composition of, 394  
 Vegetable proteins, 346  
 amino acid composition of, 346, 347, 352  
 haemoglobin and plasma protein regeneration by, 348  
 fatty livers in relation to, 350  
 vitamin B<sub>12</sub> and, 348  
 Vegmite, 310  
*Vibrio cholerae*, proteins of, immunochemical studies on, 194  
 fractionation of cells for isolating protein antigens, 194  
 heat stability of the antigens of, 195  
 gel-diffusion analysis of proteins of, 195

- adsorption of proteins of, on calcium phosphate gel, 195  
 removal of polysaccharide from protein antigens of, 195  
 cross reaction patterns of antigens of, 195  
*Vibrio El Tor* lecithinase in, characterization of, 197  
 nature of, 198  
 preparation of the lecithinase in, 197  
 excretion of lecithinase by resting and growing cells of, 198, 200  
 effect of buffer salts on excretion of, 200  
 heat stability, effect of metal ions and inhibitors on lecithinase of, 199  
 effect of sugars on synthesis of lecithinase by, 201  
 correlation between lecithinase excretion and nucleic acids in, 202  
 hemolytic activity of, in relation to lecithinase, 203  
*Vicia faba*, 375  
*Vicia hirsuta*, 432  
*Vicia sativa*, 432  
 Vitamin A, utilization, influence of dietary protein quality, 107  
 effect of different dietary proteins on blood and tissue content of rats, 109  
 effect on liver, kidney and plasma enzyme activities, 111  
 depletion on various protein diets, 110  
 binding to lipoproteins, effect of dietary proteins, 113  
 destructive factor in erythrocytes, 113  
 B-vitamins, influence on growth and regeneration of liver constituents in protein-fasted rats, 157  
 influence on regeneration of serum proteins, 159  
 Vitamin B<sub>12</sub>, influence on protein metabolism, 73  
 effect of deficiency of, on incorporation of methionine into liver protein in chicks, 74  
 distribution in sub-cellular fractions of chick liver, 74  
 reversal of KCN inhibition of phenylalanine incorporation into protein, by, 75  
 role in protein synthesis in *E. coli*, 78  
 effect on incorporation of leucine into *E. coli* proteins, 80  
 effect on purine and protein synthesis in *L. leichmanii* and *O. malhamensis*, 80  
 effect on induced synthesis of galactosidase *E. coli*, 81  
 effect of, along with folic acid, on growth and liver constituents during protein-fasting and refeeding, 160  
 effect on formation of permeases in *E. coli*, 81  
 Vitamin C effect of deficiency of, on nitrogenous tissue constituents of monkeys, 125  
 effect of deficiency on urinary excretion of NPN, 125  
 effect of deficiency on urinary excretion of tyrosyl derivatives, homogentisic acid, keto acids, tyrosine and phenylalanine, 126  
 effect on serum protein pattern in guinea pigs, 126  
 in tyrosine metabolism, 127  
 status of rat, influence of dietary protein levels, 129-132



urinary excretion and tissue levels of, in rats,  
influence of sulfa drugs, 131  
Vorlander's reagent, 47

W.H.O. protein advisory committee, suggestions  
regarding formulation of vegetable protein blends  
for human feeding, 276

## W

Weaned infants, precooked, balanced protein foods  
for, 286

Wet processing of groundnut, 263, 265

Wheat protein, 431

gluten in, 431

amino acid in, 432

protein efficiency of, 432

effect of fertilizers on, 432

## Y

Yeast hydrolysate, 319

supplementary value to rice diet, 322

growth of microorganisms on, 324

## Z

*Zea mays*, 248

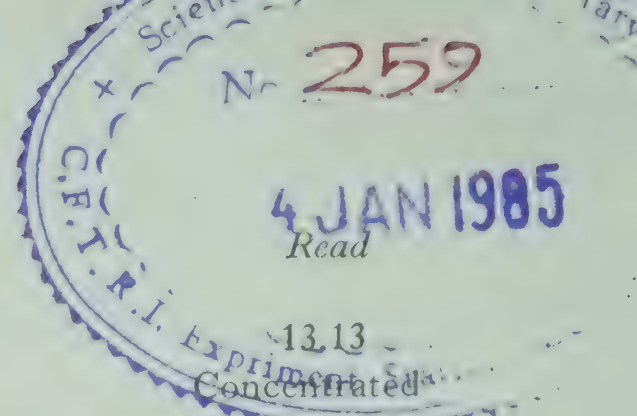
fractionation of proteins in leaf tissue of, 249

solution culture of, 248



## Errata

Page	Para	Table	Column	Line	For	Read
9		II	7	3	15.13	13.13
20	5				Concentrate	Concentrated
41		V	5	heading	fraction S <sub>2</sub>	fraction SR <sub>2</sub>
44		Fig. 1 & 2		heading	micromoles	micrograms
51		I			'%N content of' is common to columns 2, 3 & 4	
58	Reference 3				1939, 2,71,961	2,171,961 (1939);
63		II	5	4	139.6	129.6
75		III	1	2	H KCN	M KCN
80	2			1	'condition a 1'	'condition 1'
87		II	2		$\times 10^{-8}$	$\times 10^8$
88	4			2	liability	lability
91				1	$L \times o / \times t$	$L_n \times o / \times t$
118		II	6		Liver wt body wt $\times 100$	Liver wt/body wt $\times 100$
120		V	7	heading	g	mg
120		VI	3	2	149	14.9
130		I	under 'statistical significance'		I~II*, I~III†, II~III†	I~II†, I~III†, II~III*
131		II	,,		I~II*, II~IV†, II~III†	I~II†, II~IV†, II~III†
148		I	3		80 % fat	8 % fat
163			foot note,	2	200	100
165		VI		4	$\alpha$ -Tocopherol	$\alpha$ -Tocopherol 50
194	2			1	1-2	1-12
204	4			13	1.000	1,000
212		I	4	9	06	0.6
214		II	7	4	9.911	0.911
250	1			1	Folinpheno	Folin phenol
263		Fig. 3		last line	Handling losses	Handling losses 2
277		Fig. 1		13	mixture	mixer
278		II	3	2	7.5	7.6
			4	4	13	1.3
			5	3	369	36.9
			5	11	23.5	235
280		V	3	2	1.70	1.72
288		II	under 'Direct costs'		260	280
322	1			4	44	14
327		I	1	20	54	34
332		I	7 & 15	heading	S. S. P.	W. S. P.
337	1			5	17 per cent	12 per cent
340	3			3	Table V	Table VI
348		II		3	intake %	intake 10 %
353	1			5	delete 'contents of'	
356		III	9	9	2,86	2.86
362				4	glutin	glutelin
367		III			'average intake' common heading for columns 3 & 4	
369	1			7	7	6-7
370	3			2	B <sub>2</sub> -- B <sub>5</sub>	B <sub>1</sub> -- B <sub>5</sub>
374			Reference 17		p. 53	p. 539
377		II	under 'Expt III'			
			Column	3	- 45.6	- 8.2
391	1			2	B <sub>2</sub>	B <sub>12</sub>
406		I	4	2	74.5	14.5
410		IV	1	2	mg/100 g	mg/100 ml
416		VII	8	2	65.00	45.00
437	1			2	chromatography	co-chromatography





















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